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This grant proposed to determine whether organophosphate insecticides act upon the cholinergic system in the lungs increasing cholinergic neurotransmission and causing airway hyperresponsiveness, a key characteristic of asthma. Guinea pigs were treated with the organophosphate insecticides, chlorpyrifos, diazinon and parathion. Electrical stimulation of the vagus nerves caused frequency-dependent bronchoconstriction that was used to assess the state of reactivity of the airways. Pharmacologic agents were used to define the roles that specific muscarinic receptors played in the changes in airway reactivity caused by the exposures. In summary, the results of the project demonstrate that organophosphates cause airway hyperreactivity at doses lower than those that inhibit acetylcholinesterases. Thus, inhibition of acetylcholinesterase is not a good biomarker of lung toxicity. Organophosphate induced hyperreactivity is linked to loss of neuronal M2 muscarinic receptor function. Decreased M2 function leads to increased release of acetylcholine and increased bronchoconstriction. Unlike other models of hyperreactivity, loss of M2 receptor function is not mediated by eosinophils in non-sensitized guinea pigs. Furthermore, preliminary data demonstrate that atopic, sensitized, guinea pigs are sensitive to organophosphates at a 10-fold lower dose than non-sensitized animals. Thus, doses of organophosphates that have no effect in non-sensitized guinea pigs, cause hyperreactivity in sensitized guinea pigs.

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## INTRODUCTION

Over the past 20 years there has been a significant increase in the incidence of asthma in industrialized nations. Over the same timeframe, insecticide usage has increased significantly, not only in agricultural settings, but also in the inner cities. One of the most commonly used classes of insecticides is the organophosphates and a number of clinical reports and epidemiological studies have linked exposure to organophosphates to airway hyperreactivity and asthma. However, the mechanisms by which these insecticides cause changes in airway function remain unknown. In the lung, cholinergic nerves in the vagi mediate airway tone and reactivity. These nerves release acetylcholine onto M3 muscarinic receptors causing contraction of airway smooth muscle resulting in bronchoconstriction. Vagally induced bronchoconstriction is limited by autoinhibitory M2 muscarinic receptors on parasympathetic nerves. We have shown that previously that neuronal M2 receptors are dysfunctional in animal models of asthma. Loss of M2 receptor function leads to increased release of acetylcholine from the parasympathetic nerves resulting in potentiation of vagally mediated bronchoconstriction, which contributes to airway hyperreactivity. Since organophosphates are known to alter cholinergic function in the brain, we tested whether organophosphate insecticides can alter neuronal M2 muscarinic receptor function in the lungs and induce hyperreactivity.

## BODY

Three tasks were identified in the SOW of the previous CDMRP Award:

1. To determine the effect of OPs on airway responsiveness to vagal stimulation.
2. To determine the mechanism by which pesticides cause airway hyperreactivity; specifically to determine whether OPs:
  - a. Block the inhibitory function of neuronal M2 receptors
  - b. Alter the function of postjunctional M3 muscarinic receptors on airway smooth muscle
  - c. Alter acetylcholine metabolism by inhibition of AChE
3. To determine whether exposure to OPs exacerbates airway hyperreactivity following antigen challenge.

## Results:

We have completed Tasks 1 and 2. We demonstrated that exposure to each of three different OP pesticides (chlorpyrifos, parathion and diazinon) caused airway hyperreactivity as measured by increased bronchoconstriction in response to stimulation of the vagus nerves. We also demonstrated that OP-induced hyper-reactivity occurs at the level of the nerves, not at the level of airway smooth muscle, since bronchoconstriction in response to intravenous agonists (acetylcholine and methacholine) was not potentiated in animals exposed to OPs. The mechanism of action of OP-induced hyperreactivity is independent of acetylcholinesterase inhibition, and occurs by preventing the neuronal M2 receptors from inhibiting release of acetylcholine in the lungs. In addition, we demonstrated that permethrin, a non-OP pesticide, did not cause airway hyperreactivity. These data have been published in two peer-reviewed manuscripts.

We have made significant progress on Task 3, but because of initial findings, the objectives of Task 3 have been modified. To examine whether OPs exacerbate airway hyperreactivity following antigen challenge of sensitized animals we first tested controls to determine whether

sensitization alone (in the absence of inhalational challenge with antigen) altered the response to OPs. Our data demonstrate that control animals are hyperreactive to 1 mg/kg but not to 0.1 mg/kg parathion (s.c.). In contrast, sensitized animals are hyperreactive to both 0.1 and 1.0 mg/kg parathion (s.c.). Neither of these parathion concentrations inhibited AChE activity. These data demonstrate that sensitization alone is sufficient to increase sensitivity to OPs. In a separate project, we have similarly demonstrated that sensitization (without antigen challenge) also makes animals more sensitive to hyperreactivity induced by viral infection (Adamko et al 1999 J Exp Med. 190:1465) or ozone (unpublished). Together these data demonstrate that it is not antigen challenge but sensitization (allergic status) that is the important determinant of the responsiveness to subsequent stimuli that cause airway hyperreactivity. We are in the process of preparing a manuscript describing the increased sensitivity of antigen-sensitized animals to OPs, which will complete aim 3.

## **KEY RESEARCH ACCOMPLISHMENTS**

1. Chlorpyrifos, parathion, and diazinon potentiate bronchoconstriction induced by electrical stimulation of the vagi at doses that do not inhibit acetylcholinesterase. None of these organophosphates potentiate bronchoconstriction induced by intravenous methacholine, a muscarinic agonist not metabolized by acetylcholinesterase.
2. Chlorpyrifos, parathion, and diazinon all inhibit the function of inhibitory M2 receptors on the parasympathetic nerves at doses that do not inhibit acetylcholinesterase. This would increase release of acetylcholine and may be one of the mechanisms of chlorpyrifos induced hyperreactivity.
3. The ability of the organophosphates to potentiate vagally induced bronchoconstriction is dose related.
4. Sensitized guinea pigs (animals that have been sensitized to a protein, ovalbumin, but never challenged with this protein; this is a model of atopic individuals) are more sensitive to organophosphates than non-sensitized guinea pigs. Doses of parathion that have no effect in non-sensitized animals cause hyperreactivity in sensitized guinea pigs. There is no change in the ability of these compounds to interact with acetylcholinesterase in the sensitized guinea pigs.
5. Eosinophils, which are known to mediate M2 dysfunction and hyperreactivity in antigen challenged and in ozone exposed guinea pigs DO NOT contribute to organophosphate induced hyperreactivity in non-sensitized guinea pigs.
6. Eosinophils mediate organophosphate induced hyperreactivity in sensitized guinea pigs.

## **REPORTABLE OUTCOMES**

We have published two manuscripts on the effects of chlorpyrifos; both are attached as appendices. We are preparing a third manuscript on the increased sensitivity of antigen sensitized (atopic) guinea pigs to organophosphates.

Fryer AD, Lein PJ, Howard AS, Yost BL, Beckles RA, Jett DA 2004. Mechanisms of organophosphate insecticide-induced airway hyperreactivity. *Am J Physiol, Lung Cell Mol.* 286:L963-9

Lein PJ, Fryer AD. 2005 Organophosphorus insecticides induce airway hyperreactivity by decreasing neuronal M2 muscarinic receptor function independent of acetylcholinesterase inhibition. *Toxicol Sci.* 83:166-76).

## CONCLUSIONS

Organophosphates cause airway hyperreactivity at doses lower than those that inhibit acetylcholinesterases. Thus, inhibition of acetylcholinesterase is not a good biomarker of lung toxicity. Organophosphate induced hyperreactivity is linked to loss of neuronal M2 muscarinic receptor function. Decreased M2 function leads to increased release of acetylcholine and increased bronchoconstriction. Unlike other models of hyperreactivity, loss of M2 receptor function is not mediated by eosinophils in non-sensitized guinea pigs. Furthermore, preliminary data demonstrate that atopic, sensitized, guinea pigs are sensitive to organophosphates at a 10-fold lower dose than non-sensitized animals. Thus, doses of organophosphates that have no effect in non-sensitized guinea pigs, cause hyperreactivity in sensitized guinea pigs.

## Organophosphorus Insecticides Induce Airway Hyperreactivity by Decreasing Neuronal M2 Muscarinic Receptor Function Independent of Acetylcholinesterase Inhibition

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We previously demonstrated that the organophosphorus (OP) insecticide chlorpyrifos potentiates vagally induced bronchoconstriction independent of acetylcholinesterase (AChE) inhibition by decreasing the function of neuronal M2 muscarinic receptors that normally inhibit acetylcholine release from parasympathetic nerves supplying airway smooth muscle. However, it has been reported that different OPs may not affect muscarinic receptors equally. To determine if the effects of chlorpyrifos on airway hyperreactivity can be generalized to other OPs, we tested whether parathion and diazinon also inhibit neuronal M2 receptor function resulting in airway hyperreactivity. In control animals, the M2 agonist pilocarpine inhibits vagally induced bronchoconstriction in a dose-related manner. Treatment of guinea pigs with either parathion (1–10 mg/kg, sc) or diazinon (0.75–75 mg/kg, sc) shifted pilocarpine dose-response curves significantly to the right, indicating loss of neuronal M2 receptor function. These OP treatments also significantly potentiated vagally induced bronchoconstriction. Treatments that did not decrease M2 receptor function (parathion at 0.1 mg/kg, sc, or the non-OP insecticide permethrin at 150 mg/kg, sc) also did not cause airway hyperreactivity. None of the OP treatments altered bronchoconstriction induced by iv acetylcholine or methacholine in vagotomized guinea pigs, suggesting that OP-induced airway hyperreactivity is not due to altered function of muscarinic receptors on airway smooth muscle or to AChE inhibition. AChE assays of lung, blood, and brain confirmed that parathion and diazinon decreased M2 function at concentrations that did not inhibit AChE. These data suggest that multiple diethyl phosphorothionate OPs cause airway hyperreactivity via a common mechanism of M2 receptor dysfunction independent of AChE inhibition.

**Key Words:** organophosphorus pesticides; asthma; M2 muscarinic receptor; airway hyperreactivity.

Asthma prevalence and severity has increased over the past two decades, with the greatest increase occurring in children and

adolescents living in urban environments (Hartert and Peebles, 2000; Weitzman *et al.*, 1992). Over this same period, the use of insecticides, particularly organophosphorus (OP) insecticides, has increased, not only in agricultural environments (Fenske *et al.*, 2002; Koch *et al.*, 2002; USDA, 2003; Wilhoit *et al.*, 1999) but also significantly in residential and urban settings (Berkowitz *et al.*, 2003; CDC, 2003; Lu *et al.*, 2001; Weisenburger, 1993; Whyatt *et al.*, 2002). A number of clinical and epidemiological studies have linked OP exposure to symptoms associated with asthma including airway hyperreactivity and wheezing (Bryant, 1985; Deschamps *et al.*, 1994; Hoppin *et al.*, 2002; O'Malley, 1997; Salam *et al.*, 2004). The biological mechanism proposed to explain OP effects on asthma was inhibition of acetylcholinesterase (AChE, E.C. 3.1.1.7) resulting in decreased hydrolysis of acetylcholine (Casarett and Doull, 1975; Senthilselvan *et al.*, 1992), which could then increase bronchoconstriction via activation of M3 muscarinic receptors on airway smooth muscle (Coulson and Fryer, 2003; Roffel *et al.*, 1990, 1994).

In support of epidemiological evidence linking OP exposure to asthma, we have recently established that chlorpyrifos, a widely used OP, induces airway hyperreactivity in a guinea pig model (Fryer *et al.*, 2004). However, our data show that chlorpyrifos potentiates vagally induced bronchoconstriction in the absence of AChE inhibition. Rather, the mechanism involves decreased function of inhibitory M2 muscarinic receptors on the parasympathetic nerves supplying airway smooth muscle. Vagally induced bronchoconstriction normally is limited by these autoinhibitory M2 muscarinic receptors (Coulson and Fryer, 2003; Fryer and MacLagan, 1984; Minette and Barnes, 1988). Loss of M2 receptor function leads to increased release of acetylcholine from the parasympathetic nerves, resulting in potentiation of vagally mediated bronchoconstriction, which contributes to airway hyperreactivity. OP-induced inhibition of M2 receptor function and the consequent airway hyperreactivity are consistent with previous studies demonstrating that neuronal M2 receptors are dysfunctional in animal models of asthma (Fryer and Wills-Karp, 1991; Gambone *et al.*, 1994;

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Jacoby and Fryer, 1991) and in patients with asthma (Minette *et al.*, 1989).

There are reports of OPs interacting with muscarinic receptors in brain tissue at doses that do not inhibit AChE (Katz and Marquis, 1989); however, these effects appear not to be consistent across brain regions or between different OPs (Pope, 1999). Recent *in vitro* studies using slice cultures from rat striatum indicated that the active metabolite of chlorpyrifos increased acetylcholine release via inhibition of autoinhibitory muscarinic receptors (Liu *et al.*, 2002), which is consistent with our observations of chlorpyrifos effects on cholinergic neurotransmission in the airway. However, these striatal studies also found that, in the presence of AChE inhibitors, the active metabolites of the OPs parathion and methyl parathion act like muscarinic agonists to inhibit acetylcholine release. These data raise a question of whether observations of chlorpyrifos-induced airway hyperactivity via decreased M2 receptor function can be generalized across the OP class of insecticides. To address this question, we tested two different OPs, diazinon and parathion, which have different profiles of toxicity (Ecobichon, 2001; Moser, 1995). Although EPA-mandated restrictions for residential use of diazinon (Spectracide®) have recently been phased in, it remains a commonly used insecticide in the United States (USDA, 2003; Whitmore *et al.*, 2003). Moreover, there is evidence of widespread exposure to diazinon in the general population (Barr *et al.*, 2004; Whyatt *et al.*, 2002). Parathion was included in this study despite the fact that EPA cancelled all uses of this pesticide in 1992, because its toxicological properties in both humans and animals are well known, and there are previous reports that parathion induces symptoms of asthma in experimental animals (Segura *et al.*, 1999). Pyrethroids are a widely used class of non-OP pesticides that do not act via cholinesterase inhibition and have been shown to either not affect or increase muscarinic receptor function (Abou-Donia *et al.*, 2004; Ahlbom *et al.*, 1994; Eriksson and Nordberg, 1990; Husain *et al.*, 1994). For these reasons, and because there is concern that pyrethroids may exacerbate asthma (Landrigan *et al.*, 1999), we included permethrin in our tests of pesticide effects on airway hyperreactivity.

## MATERIALS AND METHODS

**Animals.** Specific pathogen-free male guinea pigs (300–350 g) were shipped from Hilltop Lab Animals Inc. (Scottsdale, PA) in filtered crates, housed in high-efficiency particulate-filtered air, and fed a normal diet (Prolab; Agway, Syracuse, NY). All protocols were approved by Animal Care and Use Committees at the Johns Hopkins and Oregon Health and Science Universities.

**Pesticide exposures.** Parathion (*o,o*-diethyl-*o*-p-nitrophenyl phosphorothioate, 99.5% pure), diazinon (*o,o*-diethyl-*o*-[2-isopropyl-4-methyl-6-pyrimidinyl] phosphorothioate, 99.5% pure), and permethrin (3-phenoxybenzyl-(1RS)-*cis*/*trans*-3,2,2-dichlorovinyl-2,2-dimethyl, 20% *cis*, 78% *trans*) were purchased from Chem Service (West Chester, PA) and used prior to the expiration date, with interim storage as recommended by the manufacturer. Pesticides dissolved in peanut oil or an equal volume (300  $\mu$ l) of peanut oil alone were administered to guinea pigs by subcutaneous (sc) injection in the subscapular region.

Subcutaneous dosing is commonly used in mechanistic studies of OPs (Bushnell *et al.*, 1991; Chiappa *et al.*, 1995; Pope *et al.*, 1992; Stanton *et al.*, 1994) and is proposed to result in gradual release of the pesticide into the systemic circulation (Pope *et al.*, 1991), which approximates most human exposures (Gallo and Lawryk, 1991). The highest doses of diazinon and parathion tested in these studies were determined to be those that caused a 50% inhibition of AChE in guinea pig lungs. The dose of permethrin used in these studies is within one order of magnitude of the amount of permethrin absorbed by guinea pig dermis (40 mg/kg) following a single application of 5% permethrin cream, which is a standard formulation for treating scabies (Franz *et al.*, 1996), and approximately one-tenth the dermal LD<sub>50</sub> reported for rats (approximately 4000 mg/kg) and rabbits (approximately 2000 mg/kg). Animals dosed with parathion or diazinon were monitored for signs of cholinergic intoxication (tremors, altered gait, and excessive excretions) at 1 and 24 h following injections. In addition, effects on physiological parameters (heart rate, blood pressure) under basal conditions were monitored in animals treated with pesticides prior to initiating experiments. Physiological measurements of lung function were carried out 24 h post injection, and since previous studies demonstrated that lung function in guinea pigs treated with peanut oil does not differ from that seen in saline-treated controls (Fryer *et al.*, 2004), only peanut oil controls are reported herein.

**Anesthesia and measurement of pulmonary inflation pressure.** Guinea pigs were anesthetized with 1.5 g/kg urethane (ip). Heart rate and blood pressure were measured from the carotid artery. The trachea was cannulated, and the animals were ventilated via a tracheal cannula with a positive pressure constant volume (1 ml per 100 g body weight and 100 breaths/minute). The jugular veins were cannulated, and the nicotinic receptor antagonist succinylcholine (10  $\mu$ g/kg/min, iv) infused to paralyze the animals. Pulmonary inflation pressure (Ppi) was measured from a side arm at the trachea; bronchoconstriction was measured as the increase in Ppi over the pressure produced by the ventilator as previously described (Fryer and MacLagan, 1984; Fryer and Wills-Karp, 1991; Jacoby and Fryer, 1991).

**Measurement of vagally induced bronchoconstriction.** All animals received guanethidine (10 mg/kg, iv) prior to the start of the experiment to deplete noradrenaline. Both vagus nerves were cut. The distal ends were placed on electrodes under oil and were stimulated at 2-min intervals (0.2 ms, 10 V, 1–25 Hz, 5-sec duration), producing frequency-dependent bronchoconstriction and bradycardia due to release of acetylcholine onto postjunctional M3 muscarinic receptors in the lungs and postjunctional M2 muscarinic receptors in the heart. Both vagally induced bronchoconstriction and bradycardia could be abolished by atropine (1 mg/kg, iv).

**Measurement of neuronal M2 muscarinic receptor function.** The function of neuronal M2 receptors was determined by measuring the ability of the muscarinic agonist, pilocarpine, to inhibit bronchoconstriction in response to vagal stimulation at 2 Hz. Pilocarpine is a muscarinic agonist with selectivity for prejunctional M2 versus postjunctional M3 receptors *in vivo* (Fryer and MacLagan, 1984; Langley, 1878), thus, pilocarpine inhibits vagally induced bronchoconstriction via stimulation of the neuronal M2 receptors at doses that are 100-fold less than the doses required to cause bronchoconstriction by stimulating postjunctional M3 receptors (Fryer and MacLagan, 1984). The effect of pilocarpine on vagally induced bronchoconstriction is reported as the ratio of bronchoconstriction in the presence of pilocarpine to bronchoconstriction in the absence of pilocarpine. A shift to the right of the pilocarpine dose-response curve indicates decreased M2 receptor function (Fryer and MacLagan, 1984; Fryer and Wills-Karp, 1991; Jacoby and Fryer, 1991).

**Measurement of postjunctional muscarinic receptor function.** Intravenous injection of acetylcholine (1–10  $\mu$ g/kg) was used to assess the function of the postjunctional M3 receptors in the lungs and postjunctional M2 receptors in the heart. To determine if AChE inhibition influenced the response to acetylcholine, these experiments were repeated using methacholine (1–10  $\mu$ g/kg, iv), an agonist that is less susceptible to hydrolysis by cholinesterases than acetylcholine (Bruning *et al.*, 1996; Norel *et al.*, 1993). Since muscarinic agonists also initiate a reflex bronchoconstriction (Delpierre *et al.*, 1983; Wagner and Jacoby, 1999), these experiments were performed in vagotomized animals.



**AChE assay.** Since OP inhibition of AChE differs between lung and brain within any given species (Lessire *et al.*, 1996), we measured AChE activity in the lung in addition to AChE activity in the brain and blood, both of which are commonly used biomarkers of OP toxicity. Immediately following the completion of physiological measurements, lungs, brain, and heparinized blood samples were collected for determination of AChE activity via the standard Ellman assay (Ellman *et al.*, 1961) using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ASChI) as the substrate. Lung and brain samples were homogenized in lysis buffer (0.1 M phosphate, pH 8.0) containing 0.1% Triton using a Dounce homogenizer, centrifuged at  $13,400 \times g$ , and the supernatant collected for analysis. Assays were run against blanks containing DTNB. The reaction was started with the addition of ASChI after equilibration for 5 min. Hydrolysis of ASChI was determined by monitoring the change in absorbance at 405 nm. To inhibit pseudocholinesterase activity, 100  $\mu$ M tetraisopropyl pyrophosphoramidate (iso-OMPA) was included in the assay. Data from lung and brain samples were normalized using protein concentration as determined using the BCA assay according to the manufacturer's directions (Pierce, Rockford, IL). AChE activity in blood samples was normalized according to the number of red blood cells (RBC) as determined using a hemacytometer.

**Statistics.** Data are expressed as mean  $\pm$  standard error of the mean (SEM). Frequency, pilocarpine, methacholine, and acetylcholine dose-response curves were analyzed using a two-way analysis of variance for repeated measures. Baseline heart rates (beats/min), blood pressures (mmHg), pulmonary inflation pressures (mmH<sub>2</sub>O), and changes in pulmonary inflation pressure (mmH<sub>2</sub>O) before pilocarpine administration/mmH<sub>2</sub>O after pilocarpine administration), as well as AChE activity levels (as % of control) were analyzed using analysis of variance (ANOVA; Statview 4.5, Abacus Concepts, Inc., Berkeley, CA); a *p* value  $\leq 0.05$  was considered significant.

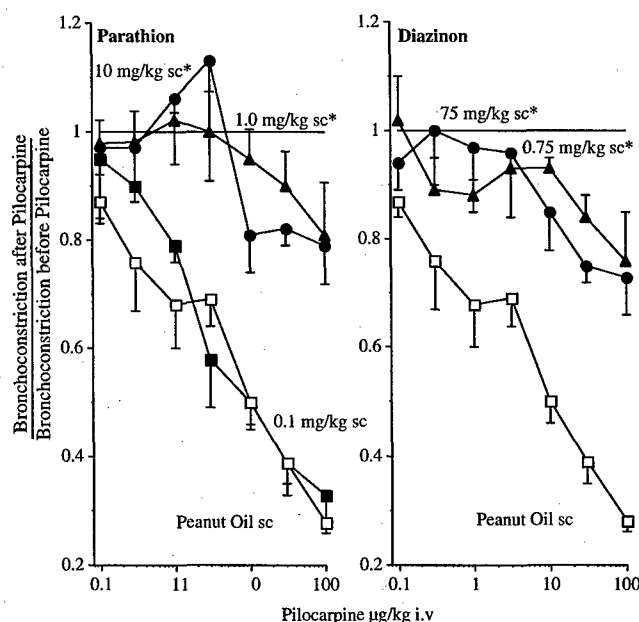
## RESULTS

Guinea pigs were injected sc with one of two OPs, parathion (0.1–10 mg/kg) or diazinon (0.75–75 mg/kg), or with the non-OP insecticide, permethrin (150 mg/kg), 24 h prior to physiological measurements. None of these treatments caused any apparent signs of cholinergic intoxication. In anesthetized, vagotomized guinea pigs, baseline pulmonary inflation pressure (Ppi) did not differ among animals treated with the highest doses of parathion, diazinon, permethrin, or the vehicle (peanut oil) control (peanut oil,  $87 \pm 8$  mmH<sub>2</sub>O; parathion,  $109 \pm 3.8$  mmH<sub>2</sub>O; diazinon,  $98 \pm 4$  mmH<sub>2</sub>O; permethrin,  $97.5 \pm 5$  mmH<sub>2</sub>O). Neither were there differences among treatment groups in resting heart rate (peanut oil,  $320.9 \pm 9$  beats/min; parathion,  $342.5 \pm 9$  beats/min; diazinon,  $321 \pm 15$  beats/min; permethrin,  $308 \pm 11$  beats/min) or in resting systolic/diastolic blood pressure (peanut oil,  $38.5 \pm 3$  mmHg/ $21 \pm 3.8$  mmHg; parathion,  $50 \pm 7.5$  mmHg/ $28 \pm 5.9$  mmHg; diazinon,  $42.5 \pm 8.8$  mmHg/ $23.5 \pm 6.4$  mmHg; permethrin,  $47.5 \pm 2$  mmHg/ $25.8 \pm 4$  mmHg) in vagotomized guinea pigs.

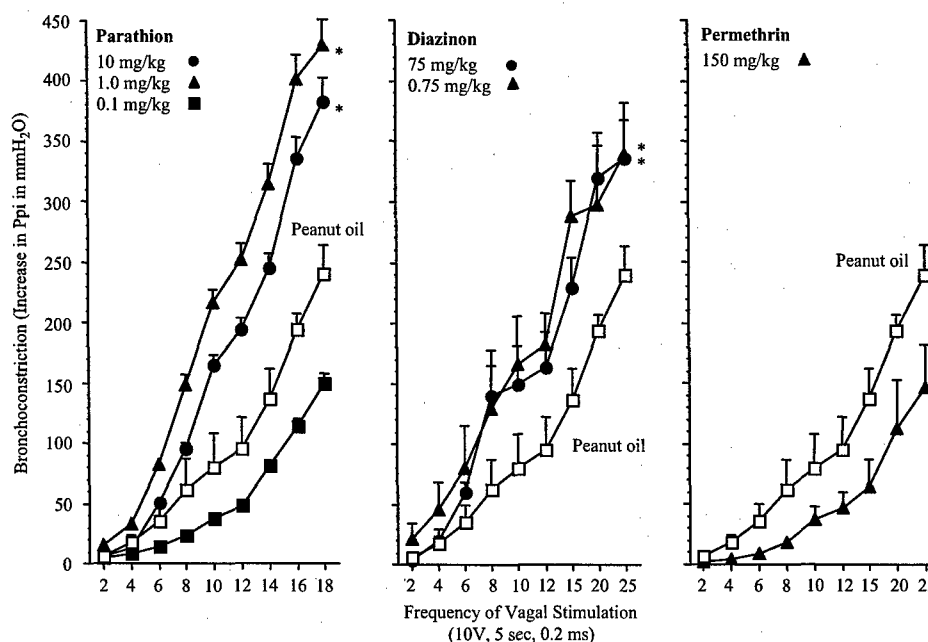
Neuronal M2 receptor function was measured using the muscarinic agonist pilocarpine. Prior to administering pilocarpine, simultaneous electrical stimulation of both vagus nerves (2 Hz, 0.2 ms, 5–15 Volts, 22 sec at 1-min intervals) produced transient bronchoconstriction (measured as an increase in Ppi) that was not different among groups (peanut oil,  $33.0 \pm 2.6$  mmH<sub>2</sub>O; parathion 10–0.1 mg/kg,  $24.2 \pm 2.7$  mmH<sub>2</sub>O,  $19.8 \pm 3$  mm H<sub>2</sub>O,  $22.1 \pm 4$  mmH<sub>2</sub>O; diazinon 75 and

0.75 mg/kg,  $32.1 \pm 3.8$  mmH<sub>2</sub>O,  $20.9 \pm 5.4$  mmH<sub>2</sub>O). In guinea pigs treated with peanut oil, pilocarpine (1–100  $\mu$ g/kg, iv) decreased vagally induced bronchoconstriction in a dose-dependent manner, demonstrating that the neuronal M2 muscarinic receptors are functional (Fig. 1, open squares). The ability of pilocarpine to decrease vagally induced bronchoconstriction was significantly inhibited in animals treated with 10 or 1.0 mg/kg, but not 0.1 mg/kg, parathion (Fig. 1, left side, filled symbols). Similarly, diazinon, at both 75 mg/kg and at a 100-fold lower dose of 0.75 mg/kg blocked the ability of pilocarpine to decrease vagally induced bronchoconstriction (Fig. 1, right side, filled symbols). Thus, both OPs inhibited the function of autoinhibitory neuronal M2 receptors.

To determine if blockade of neuronal M2 receptor function is related to airway hyperreactivity, vagally induced bronchoconstriction was measured in guinea pigs treated with these same doses of parathion and diazinon. Electrical stimulation of both vagi (1–25 Hz) caused a frequency-dependent increase in bronchoconstriction in peanut oil-treated animals (Fig. 2, open squares). Vagally induced bronchoconstriction was significantly potentiated in animals treated with parathion at 10 or 1 mg/kg, but not in animals treated with parathion at 0.1 mg/kg (Fig. 2,



**FIG. 1.** Parathion and diazinon inhibit neuronal M<sub>2</sub> receptor function. Increasing doses of pilocarpine inhibited vagally induced bronchoconstriction in a dose-related manner in animals treated with peanut oil (open squares; both panels) demonstrating functional M<sub>2</sub> receptors. In animals treated with either 1.0 or 10.0 mg/kg sc parathion (left panel), or with 0.75 or 75 mg/kg sc diazinon (right panel), pilocarpine did not inhibit vagally induced bronchoconstriction, indicating neuronal M<sub>2</sub> muscarinic receptor dysfunction. A lower dose of parathion (0.1 mg/kg, sc, closed squares, left panel) did not inhibit M<sub>2</sub> receptor function. Each point is the mean  $\pm$  SEM of four to six animals; \*significantly different from peanut oil control.



**FIG. 2.** Parathion and diazinon cause airway hyperreactivity. In vehicle treated guinea pigs (peanut oil; open squares; all panels) electrical stimulation of both vagi (1–25 Hz, 10 V, 0.2 ms, 5 s train) produced frequency-dependent bronchoconstriction, measured as an increase in pulmonary inflation pressure (Ppi). Vagally induced bronchoconstriction was significantly increased in animals treated with parathion at 1.0 and 10.0 mg/kg, sc (left panel) or with 0.75 or 75 mg/kg, sc diazinon (center panel). In contrast, treatment with either a low dose of parathion (0.1 mg/kg, sc, left panel) or with the non-OP insecticide, permethrin (right panel), did not cause airway hyperreactivity. Each point is the mean  $\pm$  SEM of four to six animals; \*significantly different from peanut oil control.

left panel, filled symbols). Both doses of diazinon potentiated vagally induced bronchoconstriction (Fig. 2, center panel, filled symbols). Thus, both OPs significantly potentiated vagally induced bronchoconstriction, but only at doses that inhibited M2 receptor function. In contrast, the non-OP insecticide, permethrin, did not potentiate vagally induced bronchoconstriction, but significantly attenuated it instead (Fig. 2, right panel, filled triangles).

To test the effect of the OPs and of permethrin on the responsiveness of airway smooth muscle and the function of the post-junctional M3 muscarinic receptors on airway smooth muscle, bronchoconstriction was induced in vagotomized guinea pigs by iv acetylcholine (Fig. 3). To test whether the response to acetylcholine was affected by OP-induced inhibition of AChE, these experiments were repeated using iv methacholine (Fig. 4), which is not rapidly metabolized by AChE. Neither the OPs, parathion and diazinon, nor the non-OP, permethrin, potentiated either acetylcholine- or methacholine-induced bronchoconstriction (Figs. 3 and 4). Thus, the OPs did not enhance the ability of the M3 receptors to respond to agonists or enhance the ability of airway smooth muscle to contract. That there was no difference between methacholine- and acetylcholine-induced bronchoconstriction would suggest that inhibition of AChE is not a mechanism for OP-induced hyperreactivity at these doses.

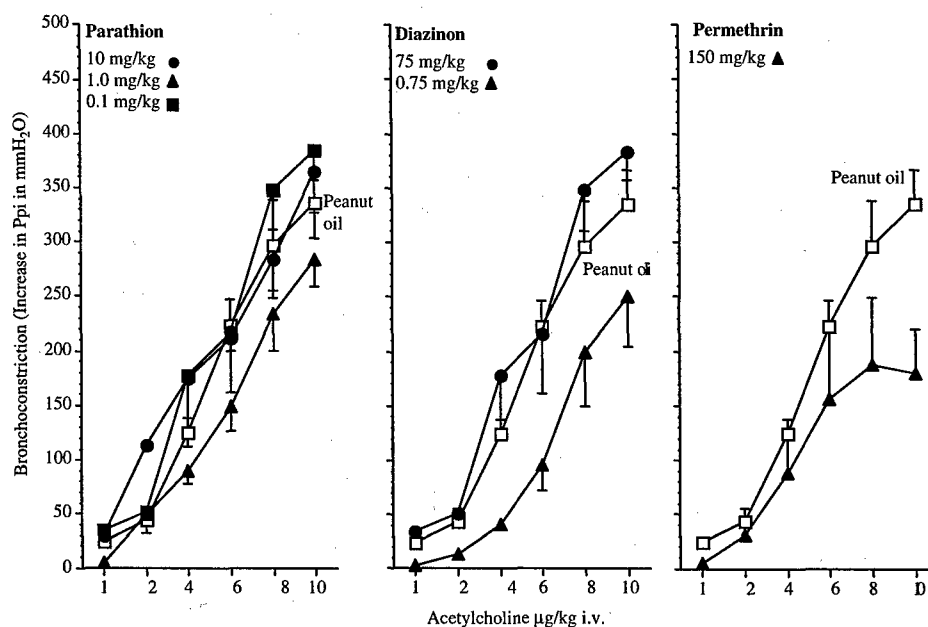
M2 muscarinic receptors are also present in the heart. Thus, we measured bradycardia to determine whether OP insecticides alter M2 muscarinic receptors in tissues other than the lung. In

the heart, stimulation of the vagus nerves (1–25 Hz) produced bradycardia that is frequency dependent (open squares, left panel, Fig. 5). Both parathion (10 mg/kg; closed circles) and diazinon (75 mg/kg; closed squares) potentiated vagally induced bradycardia (left panel, Fig. 5). In contrast, permethrin did not alter vagally induced bradycardia (closed triangles, left panel, Fig. 5). Similar to the lung, OP-induced potentiation of vagally induced bradycardia does not appear to be mediated by inhibition of AChE, since acetylcholine-induced bradycardia was not altered by either OP or by permethrin (center panel, Fig. 5). Neither was this due to any alteration in the ability of post-junctional M2 receptors to decrease heart rate, since methacholine-induced bradycardia was not different among treatment groups (right panel, Fig. 5).

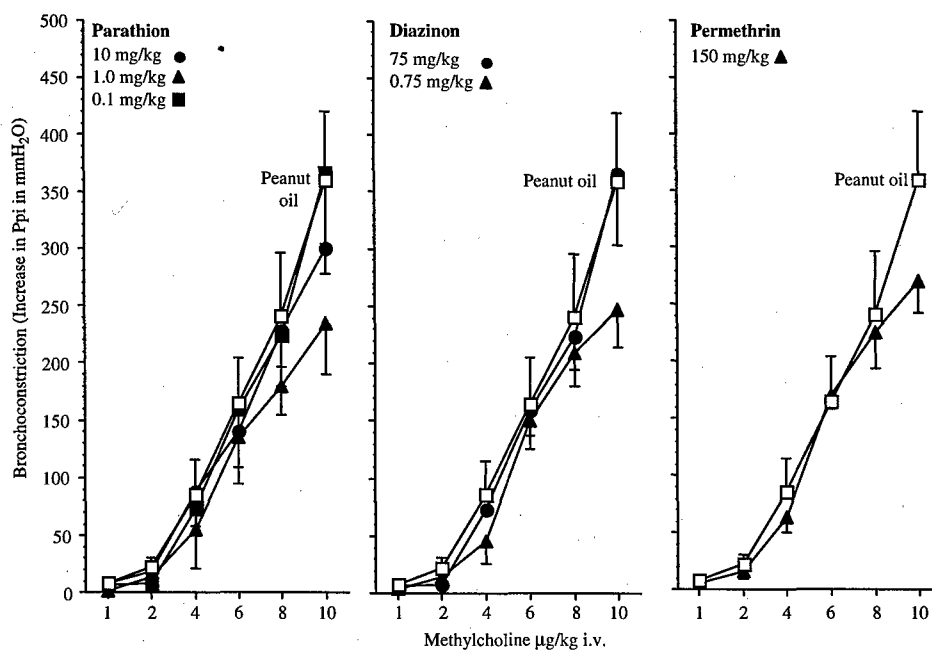
Only the highest dose of each OP significantly inhibited AChE activity in the lung and in the blood (Fig. 6). Neither 1.0 mg/kg or 0.1 mg/kg parathion or 0.75 mg/kg diazinon, or 150 mg/kg permethrin inhibited AChE activity in the periphery (Fig. 6). Brain AChE was inhibited by 10 and 1.0 mg/kg, but not by 0.1 mg/kg parathion. Neither diazinon nor permethrin inhibited brain AChE activity at the doses tested (Fig. 6).

## DISCUSSION

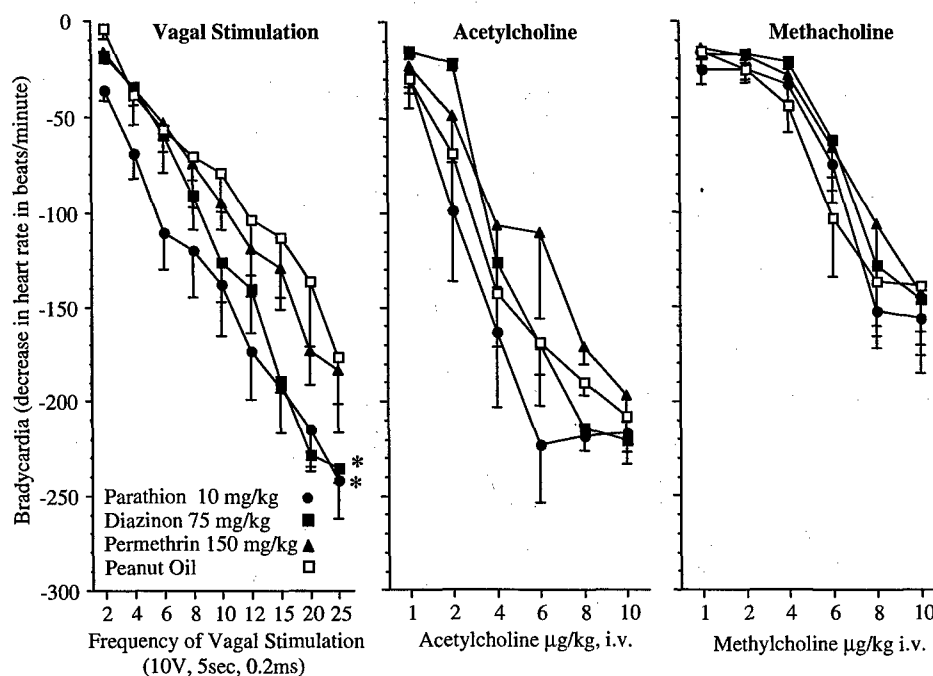
Similar to our previous observations of chlorpyrifos (Fryer *et al.*, 2004), we found that both parathion and diazinon caused



**FIG. 3.** Parathion and diazinon do not alter airway response to iv acetylcholine. Acetylcholine (1–10 μg/kg, iv) induced a dose-dependent bronchoconstriction in vagotomized guinea pigs (peanut oil controls: open circles; all panels) that was not potentiated by parathion (0.1–10.0 mg/kg, sc; filled shapes, left panel) or by diazinon (0.75–75 mg/kg, sc; filled shapes, center panel). In contrast, the non-OP insecticide, permethrin (150 mg/kg, sc; filled triangles, right panel) inhibited acetylcholine-induced bronchoconstriction. Each point is the mean  $\pm$  SEM of four to five animals; \*significantly different from peanut oil control.



**FIG. 4.** Methacholine (1–10 μg/kg, iv) induced a dose-dependent bronchoconstriction in vagotomized guinea pigs (peanut oil controls: open circles; all panels) that was not altered by parathion (filled shapes; left panel), diazinon (filled shapes; center panel), or permethrin (right panel). Each point is the mean  $\pm$  SEM of four to five animals; \*significantly different from peanut oil control.



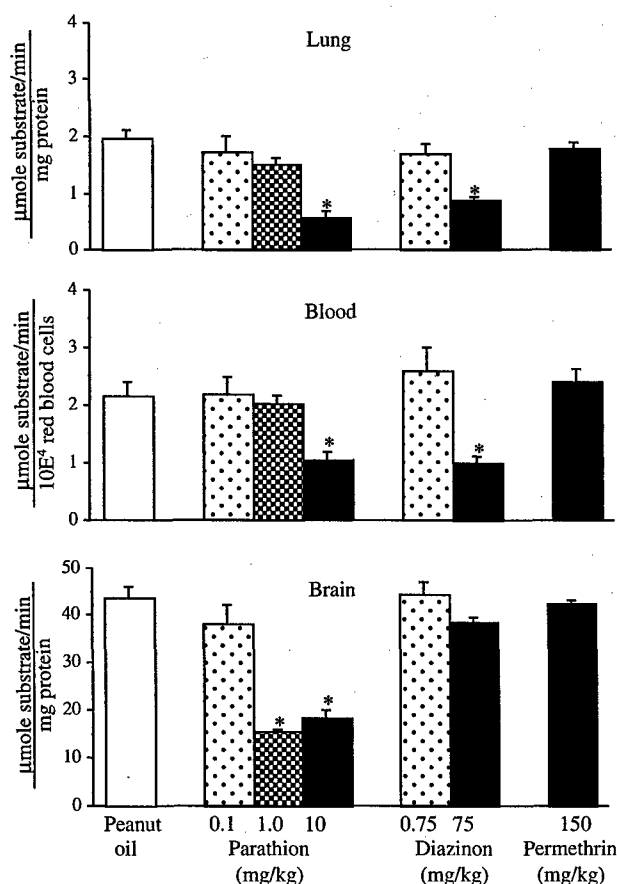
**FIG. 5.** Parathion and diazinon potentiate vagally induced bradycardia. Electrical stimulation of the vagus nerves (1–25 Hz, 10 V, 0.2 ms, 5 s train) produced frequency-dependent bradycardia measured as a fall in heart rate (open squares; left panel). Vagally induced bradycardia was significantly potentiated in animals treated with either 10 mg/kg sc parathion (filled circles; left panel) or with 75 mg/kg sc diazinon (filled squares; left panel). The non-OP insecticide permethrin had no effect on vagally induced bradycardia (filled triangles, left panel). However, OP treatment did not enhance the function of postjunctional  $M_2$  receptors, since bradycardia induced by iv acetylcholine (middle panel) or iv methylcholine (right panel) was not potentiated by parathion or diazinon. Each point is the mean  $\pm$  SEM of five to six animals; \*significantly different from peanut oil control.

airway hyperreactivity in guinea pigs, as evidenced by potentiation of vagally induced bronchoconstriction. These effects are not due to changes in either postjunctional  $M_3$  receptors or airway smooth muscle contractility, since methacholine-induced bronchoconstriction was not potentiated by either parathion or diazinon. Both doses of diazinon tested in these studies (0.75 and 75 mg/kg, sc) elicited comparable potentiation of vagally induced bronchoconstriction, and a no-effect level was not determined for this OP in these studies. However, the effects of parathion were dose-related in that the higher (1.0 and 10 mg/kg, sc) but not the lowest (0.1 mg/kg, sc) doses tested caused airway hyperreactivity. A similar dose range of parathion (3.2–17 mg/kg, ip) has been reported to increase lung resistance and to augment respiratory secretions in the rabbit lung (Segura *et al.*, 1999). In contrast to effects observed with the OPs, treatment with the non-OP insecticide, permethrin, attenuated vagally induced bronchoconstriction. Together, these data suggest that induction of airway hyperreactivity is common to diethyl phosphorothionate organophosphorus compounds, but is not a generalized property of all pesticides.

Studies of OP neurotoxicity have indicated that acute effects of these compounds are mediated primarily by AChE inhibition (Pope, 1999). Several observations from our studies rule out AChE inhibition as the mechanism underlying the potentiation of vagally induced bronchoconstriction by parathion and

diazinon. First, direct measurements of AChE activity in lungs, blood, and brain indicated that parathion and diazinon inhibited AChE in a dose-related manner, but this did not correlate with airway hyperreactivity. Second, although inhibition of AChE by pharmacological cholinesterase inhibitors has been shown to potentiate bronchoconstriction in response to acetylcholine (Colbatch and Halmagyi, 1963; Daly and Schweitzer, 1951), neither diazinon nor parathion potentiated bronchoconstriction induced by iv acetylcholine in vagotomized guinea pigs (Fig. 3), even at concentrations that caused 50% or more inhibition of AChE in the lung and blood (Fig. 6). The observation that these OPs induce airway hyperreactivity independent of AChE inhibition is important because it indicates that OP-induced airway hyperreactivity occurs below thresholds of toxic exposure that are currently defined by AChE inhibition.

In contrast, the non-OP insecticide permethrin attenuated vagally induced bronchoconstriction. Although we did not test the ability of permethrin to interact with neuronal  $M_2$  receptors, it has been reported that permethrin can increase  $M_2$  receptor function (Abou-Donia *et al.*, 2004; Ahlbom *et al.*, 1994; Eriksson and Nordberg, 1990; Husain *et al.*, 1994), which would be consistent with our observations of its effects on airway hyperreactivity. An unexpected finding was that permethrin attenuated acetylcholine-induced bronchoconstriction. The mechanism underlying this effect is not known.



**FIG. 6.** Acetylcholinesterase (AChE) activity in the lungs (upper panel), red blood cells (middle panel), and brain (lower panel) of animals treated with insecticides. Control levels are shown in open bars for peanut oil treated animals. Only the highest dose of parathion (10 mg/kg, sc) and diazinon (75 mg/kg, sc) inhibited AChE relative to control levels in the blood and lung. In the brain only parathion at the highest doses tested (1 and 10 mg/kg, sc) inhibited AChE. The non-OP insecticide, permethrin (150 mg/kg, sc), did not inhibit AChE in any of the tissues tested. Each point is the mean  $\pm$  SEM of five to eight animals; \*significantly different from control.

Neuronal M<sub>2</sub> muscarinic receptors limit release of acetylcholine from parasympathetic nerves in the lungs (Fryer and MacLagan, 1984). Pharmacological blockade of neuronal M<sub>2</sub> receptors increases release of acetylcholine from these nerves (Baker *et al.*, 1992; Fryer *et al.*, 1996), which potentiates vagally induced bronchoconstriction (Fryer and MacLagan, 1984; Fryer and Wills-Karp, 1991; Jacoby and Fryer, 1991). Our data show that neuronal M<sub>2</sub> receptor function is inhibited by both parathion and diazinon at doses that cause airway hyperreactivity. In contrast, a dose of parathion that does not affect M<sub>2</sub> receptor function also does not alter vagally induced bronchoconstriction. Thus, OP-induced inhibition of neuronal M<sub>2</sub> receptor function mediates airway hyperreactivity. Loss of neuronal M<sub>2</sub> receptor function in the lungs is also associated with other models of

airway hyperreactivity including antigen challenge (Fryer and Wills-Karp, 1991), viral infection (Jacoby and Fryer, 1991), and exposure to ozone (Gambone *et al.*, 1994), suggesting that decreased M<sub>2</sub> receptor function on airway nerves is a generalized mechanism underlying airway hyperreactivity.

The ability of OPs to inhibit neuronal M<sub>2</sub> receptors may not be restricted to the lungs. OPs compete for binding to muscarinic receptors in the brain (Abdallah *et al.*, 1992; Bomser and Casida, 2001; Huff *et al.*, 1994; Jett *et al.*, 1993, 1994; Katz and Marquis, 1989, 1992) and in the heart (Silveira *et al.*, 1990). In the heart, M<sub>2</sub> receptors are present on both cardiac muscle, where they mediate bradycardia (Brodde *et al.*, 2001; Maeda *et al.*, 1988), and parasympathetic nerves that supply the heart, where they function to inhibit release of acetylcholine (Manabe *et al.*, 1991; Oberhauser *et al.*, 2001). Both parathion (10 mg/kg, sc) and diazinon (75 mg/kg, sc) potentiated bradycardia induced by vagal stimulation but not bradycardia induced by iv administration of acetylcholine or methacholine, suggesting inhibition of prejunctional neuronal M<sub>2</sub> receptors, but not postjunctional cardiac M<sub>2</sub> receptors. Similarly, the function of the neuronal M<sub>2</sub>, but not postjunctional M<sub>2</sub>, receptors in the heart is inhibited by chlorpyrifos (Fryer *et al.*, 2004) and by systemic administration of double-stranded RNA (Bowerfind *et al.*, 2002). Thus neuronal M<sub>2</sub> receptors appear to be more vulnerable to inhibition than postjunctional M<sub>2</sub> receptors.

The interaction of OPs with pre- and postjunctional muscarinic receptors in the brain is complex. OPs have been demonstrated to antagonize muscarinic receptors either via direct effects on the receptors themselves (Fitzgerald and Costa, 1992; Huff *et al.*, 2001; Katz and Marquis, 1989; Liu *et al.*, 2002; Zhu *et al.*, 1991) or indirectly by decreasing receptor number in response to increased acetylcholine resulting from AChE inhibition (Cioffi and el-Fakahany, 1986; Zhu *et al.*, 1991). Conversely, it has also been reported that OPs stimulate neuronal muscarinic receptors either directly (Liu *et al.*, 2002; Ward and Mundy, 1996) or indirectly as a result of increased synaptic levels of acetylcholine consequent to AChE inhibition (Kilbinger and Wessler, 1980; Liu *et al.*, 2002). Reports of OPs directly stimulating muscarinic receptors were derived from functional studies of striatum (Liu *et al.*, 2002) and frontal cortex (Ward and Mundy, 1996). Although there are M<sub>2</sub> receptors on presynaptic nerves in both the striatum (Hersch *et al.*, 1994) and cortex (Levey *et al.*, 1991), studies in knockout mice suggest that it is the M<sub>4</sub>, and not the M<sub>2</sub> receptors, that are functionally significant in inhibiting acetylcholine release in these brain regions (Zhang *et al.*, 2002). These observations raise the possibility that earlier reports of direct stimulation of muscarinic receptors by OPs reflect effects on M<sub>4</sub> rather than M<sub>2</sub> receptors. When considered together with our findings that OPs inhibit neuronal M<sub>2</sub> receptor function in the lungs and heart, these data strongly suggest that M<sub>2</sub> and M<sub>4</sub> receptors are differentially affected by OPs.

The mechanism(s) by which OPs inhibit M<sub>2</sub> receptor function are not yet known. Because parathion and diazinon decreased

M2 receptor function at doses that did not inhibit AChE, it seems unlikely that either indirect stimulation or decreased expression of M2 receptors secondary to AChE inhibition underlie M2 receptor inhibition. Although it is possible that AChE was acutely inhibited at the time of administration, causing persistent downregulation of M2 receptors 24 h later, this seems unlikely because the function of the postjunctional M2 receptors on the heart (see Fig. 5) was not inhibited 24 h after administration of OPs. Furthermore, the OPs were administered not as a single bolus dose, but rather subcutaneously in oil, a method that allows for gradual release of the OPs in the systemic circulation (Pope *et al.*, 1991). It is also not the case that M4 receptors mediate OP-induced airway hyperreactivity, since M4 receptors are not expressed on parasympathetic nerves in the lungs (Fryer *et al.*, 1996). Therefore, it seems likely that OPs directly inhibit neuronal M2 receptor function. Whether they do so by downregulation of muscarinic receptor expression (Jett *et al.*, 1993, 1994), modulation of ligand binding to M2 receptors (Jett *et al.*, 1991; Katz and Marquis, 1989, 1992), or alteration of signal transduction pathways downstream of muscarinic receptor (Bomser *et al.*, 2002; Huff *et al.*, 1994; Schuh *et al.*, 2002; Ward and Mundy, 1996) has yet to be determined. A significant difference between these earlier published studies and our findings is that the former reported the potency of OP binding to muscarinic receptors as comparable to that of OP binding to acetylcholinesterase (AChE), whereas our data suggest that OP interactions with neuronal M2 receptors in airways occur at lower doses than those required to inhibit AChE activity in the lung or blood.

Data presented here confirm that the diethyl phosphorothionate OP insecticides cause airway hyperreactivity via a common mechanism of disrupting negative feedback control of cholinergic regulation in the lungs. Thus, we have shown that not only chlorpyrifos (Fryer *et al.*, 2004), but also diazinon and parathion, inhibit neuronal M2 receptor function in the lung at concentrations that do not inhibit AChE. Since the resting vagal tone in guinea pig lungs is approximately 10–15 Hz (Myers and Undem, 1996), our data suggest that loss of M2 receptor function results in increased basal release of acetylcholine. Furthermore, irritation of the lung results in reflex bronchoconstriction that is mediated by the parasympathetic nerves (Carr and Undem, 2003; Undem and Carr, 2002), and loss of M2 receptor function also increases reflex bronchoconstriction (Costello *et al.*, 1999; Evans *et al.*, 2000). M2 receptors on parasympathetic nerves supplying glands in the airways also regulate mucin secretion in the airways (Ramnarine *et al.*, 1996; Rogers, 2001). Thus OPs could potentiate basal tone, reflex bronchoconstriction, and mucus secretion, all of which are characteristics of asthma.

Use of OP insecticides has increased significantly in urban and agricultural settings over the past 30 years (Fenske *et al.*, 2002; Koch *et al.*, 2002; USDA, 2003; Wilhoit *et al.*, 1999), coincident with an increase in asthma (Hartert and Peebles, 2000; Weitzman *et al.*, 1992). Children represent a potentially sensitive subpopulation with respect to asthma, and there is evidence of wide-spread exposure of children to OPs. Screens of

fetal exposure to OP pesticides have detected chlorpyrifos (8.26 µg/ml), diazinon (13 µg/ml), and parathion (2.3 µg/ml) in meconium (Ostrea *et al.*, 2002). Recent studies in Seattle found that of 110 preschool children from 96 households of varying cultures, family income, and housing type, all excreted OP metabolites in their urine (Curl *et al.*, 2003; Lu *et al.*, 2001). Similarly, in a sample of 84,000 children across the United States, the urinary levels of chlorpyrifos metabolites were above the detection limit 98% of the time, compared to a 4% detection rate for the herbicide atrazine (Adgate *et al.*, 2001). Not only is there widespread exposure of children to these insecticides, but data collected as part of the most recent National Health and Nutrition Examination Survey (NHANES) indicated that across all racial and ethnic groups, urinary concentrations of OP metabolites in children 6–11 years of age were consistently significantly higher than in adults (Barr *et al.*, 2004). Consistent with these conclusions, chlorpyrifos residues have been shown to persist in the home for up to 2 weeks after a single application, with potential exposure to infants and children reaching levels 60–120 times greater than the U.S. EPA recommended reference levels (Fenske *et al.*, 1990; Gurunathan *et al.*, 1998). The U.S. EPA has determined the dermal and acute dietary NOAEL (no observed adverse effect level) for diazinon to be 1 mg/kg/day and 0.25 mg/kg/day, respectively, based on plasma cholinesterase inhibition (U.S. EPA, 2000). We observed airway hyperreactivity in response to diazinon (sc) at a dose that did not inhibit plasma cholinesterase, suggesting that diazinon may exert adverse effects on human lung function at doses considered safe under current EPA guidelines. These data suggest that exposure to these compounds may contribute to the observed increase in asthma prevalence over the past 30 years (Hartert and Peebles, 2000; Weitzman *et al.*, 1992).

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## REFERENCES

- Abdallah, E. A., Jett, D. A., Eldefrawi, M. E., and Eldefrawi, A. T. (1992). Differential effects of paraoxon on the M3 muscarinic receptor and its effector system in rat submaxillary gland cells. *J. Biochem. Toxicol.* **7**, 125–132.
- Abou-Donia, M. B., Dechkovskaia, A. M., Goldstein, L. B., Abdel-Rahman, A., Bullman, S. L., and Khan, W. A. (2004). Co-exposure to pyridostigmine bromide, DEET, and/or permethrin causes sensorimotor deficit and alterations in brain acetylcholinesterase activity. *Pharmacol. Biochem. Behav.* **77**, 253–262.
- Adgate, J. L., Barr, D. B., Clayton, C. A., Eberly, L. E., Freeman, N. C., Li, P. J., Needham, L. L., Pellizzari, E. D., Quackenboss, J. J., Roy, A., *et al.* (2001). Measurement of children's exposure to pesticides: Analysis of urinary metabolite levels in a probability-based sample. *Environ. Health Perspect.* **109**, 583–590.
- Ahlborn, J., Fredriksson, A., and Eriksson, P. (1994). Neonatal exposure to a type-I pyrethroid (bioallethrin) induces dose-response changes in brain

- muscarinic receptors and behaviour in neonatal and adult mice. *Brain Res.* **645**, 318–324.
- Baker, D. G., Don, H. F., and Brown, J. K. (1992). Direct measurement of acetylcholine release in guinea pig trachea. *Am. J. Physiol.* **263**, L142–L147.
- Barr, D. B., Bravo, R., Weerasekera, G., Caltabiano, L. M., Whitehead, R. D., Jr., Olsson, A. O., Caudill, S. P., Schober, S. E., Pirkle, J. L., Sampson, E. J., et al. (2004). Concentrations of dialkyl phosphate metabolites of organophosphorus pesticides in the U.S. population. *Environ. Health Perspect.* **112**, 186–200.
- Berkowitz, G. S., Obel, J., Deych, E., Lapinski, R., Godbold, J., Liu, Z., Landrigan, P. J., and Wolff, M. S. (2003). Exposure to indoor pesticides during pregnancy in a multiethnic, urban cohort. *Environ. Health Perspect.* **111**, 79–84.
- Bomser, J. A., and Casida, J. E. (2001). Diethylphosphorylation of rat cardiac M2 muscarinic receptor by chlorpyrifos oxon *in vitro*. *Toxicol. Lett.* **119**, 21–26.
- Bomser, J. A., Quistad, G. B., and Casida, J. E. (2002). Chlorpyrifos oxon potentiates diacylglycerol-induced extracellular signal-regulated kinase (ERK 44/42) activation, possibly by diacylglycerol lipase inhibition. *Toxicol. Appl. Pharmacol.* **178**, 29–36.
- Bowerfind, W. M., Fryer, A. D., and Jacoby, D. B. (2002). Double-stranded RNA causes airway hyperreactivity and neuronal M2 muscarinic receptor dysfunction. *J. Appl. Physiol.* **92**, 1417–1422.
- Brodde, O. E., Bruck, H., Leineweber, K., and Seyfarth, T. (2001). Presence, distribution and physiological function of adrenergic and muscarinic receptor subtypes in the human heart. *Basic Res. Cardiol.* **96**, 528–538.
- Bruning, T. A., Chang, P. C., Kemme, M. J., Vermeij, P., Pffaffendorf, M., and van Zwieten, P. A. (1996). Comparison of cholinergic vasodilator responses to acetylcholine and methacholine in the human forearm. *Blood Press.* **5**, 333–341.
- Bryant, D. H. (1985). Asthma due to insecticide sensitivity. *Aust. N.Z. J. Med.* **15**, 66–68.
- Bushnell, P. J., Padilla, S. S., Ward, T., Pope, C. N., and Olszyk, V. B. (1991). Behavioral and neurochemical changes in rats dosed repeatedly with diisopropylfluorophosphate. *J. Pharmacol. Exp. Ther.* **256**, 741–750.
- Carr, M. J., and Undem, B. J. (2003). Pharmacology of vagal afferent nerve activity in guinea pig airways. *Pulm. Pharmacol. Ther.* **16**, 45–52.
- Casarett, L. J., and Doull, J. (1975). *Toxicology*. Macmillan, New York, NY.
- CDC (2003). Second National Report on Human Exposure to Environmental Chemicals. NCEH Pub. No. 03-0022. National Center for Environmental Health.
- Chiappa, S., Padilla, S., Koenigsberger, C., Moser, V., and Brimijoin, S. (1995). Slow accumulation of acetylcholinesterase in rat brain during enzyme inhibition by repeated dosing with chlorpyrifos. *Biochem. Pharmacol.* **49**, 955–963.
- Cioffi, C. L., and el-Fakahany, E. E. (1986). Decreased binding of the muscarinic antagonist [3H]N-methylscopolamine in mouse brain following acute treatment with an organophosphate. *Eur. J. Pharmacol.* **132**, 147–154.
- Colbatch, H. J. H., and Halmagyi, D. F. J. (1963). Effect of vagotomy and vagal stimulation on lung mechanics and circulation. *J. Appl. Physiol.* **18**, 881–887.
- Costello, R. W., Evans, C. M., Yost, B. L., Belmonte, K. E., Gleich, G. J., Jacoby, D. B., and Fryer, A. D. (1999). Antigen-induced hyperreactivity to histamine: Role of the vagus nerves and eosinophils. *Am. J. Physiol.* **276**, L709–L714.
- Coulson, F. R., and Fryer, A. D. (2003). Muscarinic acetylcholine receptors and airway diseases. *Pharmacol. Ther.* **98**, 59–69.
- Curl, C. L., Fenske, R. A., and Elgethun, K. (2003). Organophosphorus pesticide exposure of urban and suburban preschool children with organic and conventional diets. *Environ. Health Perspect.* **111**, 377–382.
- Daly, M. d. B., and Schweitzer, A. (1951). Reflex bronchomotor responses to stimulation of receptors in the regions of the carotid sinus and aortic arch in the dog and cat. *J. Physiol. (London)* **113**, 442–462.
- Delpierre, S., Orehek, J., Beaupre, A., Velardocchio, J. M., Fornaris, M., and Grimaud, C. (1983). Comparative reflex action of histamine, acetylcholine and serotonin on dog airways. *Bull. Eur. Physiopathol. Respir.* **19**, 489–493.
- Deschamps, D., Questel, F., Baud, F. J., Gervais, P., and Dally, S. (1994). Persistent asthma after acute inhalation of organophosphate insecticide. *Lancet* **344**, 1712.
- Ecobichon, D. J. (2001). Toxic effects of pesticides. In *Casarett and Doull's Toxicology: The Basic Science of Poisons* (C. D. Klaassen, Ed.), pp. 763–810. McGraw-Hill, New York.
- Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.
- Eriksson, P., and Nordberg, A. (1990). Effects of two pyrethroids, bioallethrin and deltamethrin, on subpopulations of muscarinic and nicotinic receptors in the neonatal mouse brain. *Toxicol. Appl. Pharmacol.* **102**, 456–463.
- Evans, C. M., Belmonte, K. E., Costello, R. W., Jacoby, D. B., Gleich, G. J., and Fryer, A. D. (2000). Substance P-induced airway hyperreactivity is mediated by neuronal M(2) receptor dysfunction. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **279**, L477–L486.
- Fenske, R. A., Black, K. G., Elkner, K. P., Lee, C. L., Methner, M. M., and Soto, R. (1990). Potential exposure and health risks of infants following indoor residential pesticide applications. *Am. J. Public Health* **80**, 689–693.
- Fenske, R. A., Lu, C., Barr, D., and Needham, L. (2002). Children's exposure to chlorpyrifos and parathion in an agricultural community in central Washington State. *Environ. Health Perspect.* **110**, 549–553.
- Fitzgerald, B. B., and Costa, L. G. (1992). Modulation of M1 and M2 muscarinic receptor subtypes following repeated organophosphate exposure in rats. *Toxicol. Appl. Pharmacol.* **117**, 122–125.
- Franz, T. J., Lehman, P. A., Franz, S. F., and Guin, J. D. (1996). Comparative percutaneous absorption of lindane and permethrin. *Arch. Dermatol.* **132**, 901–905.
- Fryer, A. D., Elbon, C. L., Kim, A. L., Xiao, H. Q., Levey, A. I., and Jacoby, D. B. (1996). Cultures of airway parasympathetic nerves express functional M2 muscarinic receptors. *Am. J. Respir. Cell Mol. Biol.* **15**, 716–725.
- Fryer, A. D., Lein, P. J., Howard, A. S., Yost, B. L., Beckles, R. A., and Jett, D. A. (2004). Mechanisms of organophosphate insecticide-induced airway hyperreactivity. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **286**, L963–L969.
- Fryer, A. D., and MacLagan, J. (1984). Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br. J. Pharmacol.* **83**, 973–978.
- Fryer, A. D., and Wills-Karp, M. (1991). Dysfunction of M2-muscarinic receptors in pulmonary parasympathetic nerves after antigen challenge. *J. Appl. Physiol.* **71**, 2255–2261.
- Gallo, M. A., and Lawryk, N. J. (1991). Organic phosphorus pesticides. In *Handbook of Pesticide Toxicology* (W. J. Hayes and E. R. Laws, Eds.), Vol. 2, pp. 917–972. Academic Press, New York, NY.
- Gambone, L. M., Elbon, C. L., and Fryer, A. D. (1994). Ozone-induced loss of neuronal M2 muscarinic receptor function is prevented by cyclophosphamide. *J. Appl. Physiol.* **77**, 1492–1499.
- Gurunathan, S., Robson, M., Freeman, N., Buckley, B., Roy, A., Meyer, R., Bukowski, J., and Liroy, P. J. (1998). Accumulation of chlorpyrifos on residential surfaces and toys accessible to children. *Environ. Health Perspect.* **106**, 9–16.
- Hartert, T. V., and Peebles, R. S., Jr. (2000). Epidemiology of asthma: The year in review. *Curr. Opin. Pulm. Med.* **6**, 4–9.
- Hersch, S. M., Gutekunst, C. A., Rees, H. D., Heilman, C. J., and Levey, A. I. (1994). Distribution of m1-m4 muscarinic receptor proteins in the rat striatum: Light and electron microscopic immunocytochemistry using subtype-specific antibodies. *J. Neurosci.* **14**, 3351–3363.

- Hoppin, J. A., Umbach, D. M., London, S. J., Alavanja, M. C., and Sandler, D. P. (2002). Chemical predictors of wheeze among farmer pesticide applicators in the Agricultural Health Study. *Am. J. Respir. Crit. Care Med.* **165**, 683–689.
- Huff, R. A., Abu-Qare, A. W., and Abou-Donia, M. B. (2001). Effects of subchronic *in vivo* chlorpyrifos exposure on muscarinic receptors and adenylate cyclase of rat striatum. *Arch. Toxicol.* **75**, 480–486.
- Huff, R. A., Corcoran, J. J., Anderson, J. K., and Abou-Donia, M. B. (1994). Chlorpyrifos oxon binds directly to muscarinic receptors and inhibits cAMP accumulation in rat striatum. *J. Pharmacol. Exp. Ther.* **269**, 329–335.
- Husain, R., Malaviya, M., and Seth, P. K. (1994). Effect of deltamethrin on regional brain polyamines and behaviour in young rats. *Pharmacol. Toxicol.* **74**, 211–215.
- Jacoby, D. B., and Fryer, A. D. (1991). Virus-induced airway hyperresponsiveness—possible involvement of neural mechanisms. *Am. Rev. Respir. Dis.* **144**, 1422–1423.
- Jett, D. A., Abdallah, E. A., El-Fakahany, E. E., Eldefrawi, M. E., and Eldefrawi, A. T. (1991). The high affinity activation by paroxon of a muscarinic receptor subtype in rat brain striatum. *Pest. Biochem. Physiol.* **39**, 149–157.
- Jett, D. A., Fernando, J. C., Eldefrawi, M. E., and Eldefrawi, A. T. (1994). Differential regulation of muscarinic receptor subtypes in rat brain regions by repeated injections of parathion. *Toxicol. Lett.* **73**, 33–41.
- Jett, D. A., Hill, E. F., Fernando, J. C., Eldefrawi, M. E., and Eldefrawi, A. T. (1993). Down-regulation of muscarinic receptors and the m3 subtype in white-footed mice by dietary exposure to parathion. *J. Toxicol. Environ. Health* **39**, 395–415.
- Katz, L. S., and Marquis, J. K. (1989). Modulation of central muscarinic receptor binding *in vitro* by ultralow levels of the organophosphate paraoxon. *Toxicol. Appl. Pharmacol.* **101**, 114–123.
- Katz, L. S., and Marquis, J. K. (1992). Organophosphate-induced alterations in muscarinic receptor binding and phosphoinositide hydrolysis in the human SK-N-SH cell line. *Neurotoxicology* **13**, 365–378.
- Kilbinger, H., and Wessler, I. (1980). Inhibition by acetylcholine of the stimulation-evoked release of  $[^3H]$ acetylcholine from the guinea-pig myenteric plexus. *Neuroscience* **5**, 1331–1340.
- Koch, D., Lu, C., Fisker-Andersen, J., Jolley, L., and Fenske, R. A. (2002). Temporal association of children's pesticide exposure and agricultural spraying: Report of a longitudinal biological monitoring study. *Environ. Health Perspect.* **110**, 829–833.
- Landrigan, P. J., Claudio, L., Markowitz, S. B., Berkowitz, G. S., Brenner, B. L., Romero, H., Wetmur, J. G., Matte, T. D., Gore, A. C., Godbold, J. H., et al. (1999). Pesticides and inner-city children: Exposures, risks, and prevention. *Environ. Health Perspect.* **107**(Suppl. 3), 431–437.
- Langley, J. N. (1878). On the physiology of the salivary secretion. *J. Physiol.* **1**, 339–369.
- Lessire, F., Gustin, P., Delaunois, A., Bloden, S., Nemmar, A., Vargas, M., and Ansay, M. (1996). Relationship between parathion and paraoxon toxicokinetics, lung metabolic activity, and cholinesterase inhibition in guinea pig and rabbit lungs. *Toxicol. Appl. Pharmacol.* **138**, 201–210.
- Levey, A. I., Kitt, C. A., Simonds, W. F., Price, D. L., and Brann, M. R. (1991). Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J. Neurosci.* **11**, 3218–3226.
- Liu, J., Chakraborti, T., and Pope, C. (2002). *In vitro* effects of organophosphorus anticholinesterases on muscarinic receptor-mediated inhibition of acetylcholine release in rat striatum. *Toxicol. Appl. Pharmacol.* **178**, 102–108.
- Lu, C., Knutson, D. E., Fisker-Andersen, J., and Fenske, R. A. (2001). Biological monitoring survey of organophosphorus pesticide exposure among pre-school children in the Seattle metropolitan area. *Environ. Health Perspect.* **109**, 299–303.
- Maeda, A., Kubo, T., Mishina, M., and Numa, S. (1988). Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes. *FEBS Lett.* **239**, 339–342.
- Manabe, N., Foldes, F. F., Torocsik, A., Nagashima, H., Goldiner, P. L., and Vizi, E. S. (1991). Presynaptic interaction between vagal and sympathetic innervation in the heart: Modulation of acetylcholine and noradrenaline release. *J. Auton. Nerv. Syst.* **32**, 233–242.
- Minette, P. A., and Barnes, P. J. (1988). Prejunctional inhibitory muscarinic receptors on cholinergic nerves in human and guinea pig airways. *J. Appl. Physiol.* **64**, 2532–2537.
- Minette, P. A., Lammers, J. W., Dixon, C. M., McCusker, M. T., and Barnes, P. J. (1989). A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. *J. Appl. Physiol.* **67**, 2461–2465.
- Moser, V. C. (1995). Comparisons of the acute effects of cholinesterase inhibitors using a neurobehavioral screening battery in rats. *Neurotoxicol. Teratol.* **17**, 617–625.
- Myers, A. C., and Undem, B. J. (1996). Muscarinic receptor regulation of synaptic transmission in airway parasympathetic ganglia. *Am. J. Physiol.* **270**, L630–L636.
- Norel, X., Angrisani, M., Labat, C., Gorenne, I., Dulmet, E., Rossi, F., and Brink, C. (1993). Degradation of acetylcholine in human airways: Role of butyrylcholinesterase. *Br. J. Pharmacol.* **108**, 914–919.
- Oberhauser, V., Schwertfeger, E., Rutz, T., Beyersdorf, F., and Rump, L. C. (2001). Acetylcholine release in human heart atrium: Influence of muscarinic autoreceptors, diabetes, and age. *Circulation* **103**, 1638–1643.
- O'Malley, M. (1997). Clinical evaluation of pesticide exposure and poisonings. *Lancet* **349**, 1161–1166.
- Ostrea, E. M., Morales, V., Ngoumna, E., Prescilla, R., Tan, E., Hernandez, E., Ramirez, G. B., Cifra, H. L., and Manlapaz, M. L. (2002). Prevalence of fetal exposure to environmental toxins as determined by meconium analysis. *Neurotoxicology* **23**, 329–339.
- Pope, C. N. (1999). Organophosphorus pesticides: Do they all have the same mechanism of toxicity? *J. Toxicol. Environ. Health B Crit. Rev.* **2**, 161–181.
- Pope, C. N., Chakraborti, T. K., Chapman, M. L., and Farrar, J. D. (1992). Long-term neurochemical and behavioral effects induced by acute chlorpyrifos treatment. *Pharmacol. Biochem. Behav.* **42**, 251–256.
- Pope, C. N., Chakraborti, T. K., Chapman, M. L., Farrar, J. D., and Arthun, D. (1991). Comparison of *in vivo* cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. *Toxicology* **68**, 51–61.
- Ramnarine, S. I., Haddad, E. B., Khawaja, A. M., Mak, J. C., and Rogers, D. F. (1996). On muscarinic control of neurogenic mucus secretion in ferret trachea. *J. Physiol.* **494**(2), 577–586.
- Roffel, A. F., Elzinga, C. R., and Zaagsma, J. (1990). Muscarinic M3 receptors mediate contraction of human central and peripheral airway smooth muscle. *Pulm. Pharmacol.* **3**, 47–51.
- Roffel, A. F., Meurs, H., and Zaagsma, J. (1994). Muscarinic acetylcholine receptors and control of smooth muscle tone. *Trends Pharmacol. Sci.* **15**, 407–408.
- Rogers, D. F. (2001). Motor control of airway goblet cells and glands. *Respir. Physiol.* **125**, 129–144.
- Salam, M. T., Li, Y. F., Langholz, B., and Gilliland, F. D. (2004). Early-life environmental risk factors for asthma: Findings from the Children's Health Study. *Environ. Health Perspect.* **112**, 760–765.
- Schuh, R. A., Lein, P. J., Beckles, R. A., and Jett, D. A. (2002). Noncholinesterase mechanisms of chlorpyrifos neurotoxicity: Altered phosphorylation of Ca<sup>2+</sup>/cAMP response element binding protein in cultured neurons. *Toxicol. Appl. Pharmacol.* **182**, 176–185.
- Segura, P., Chavez, J., Montano, L. M., Vargas, M. H., Delaunois, A., Carbajal, V., and Gustin, P. (1999). Identification of mechanisms involved in the acute airway toxicity induced by parathion. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **360**, 699–710.
- Senthilselvan, A., McDuffie, H. H., and Dosman, J. A. (1992). Association of asthma with use of pesticides. Results of a cross-sectional survey of farmers. *Am. Rev. Respir. Dis.* **146**, 884–887.



- Silveira, C. L., Eldefrawi, A. T., and Eldefrawi, M. E. (1990). Putative M2 muscarinic receptors of rat heart have high affinity for organophosphorus anticholinesterases. *Toxicol. Appl. Pharmacol.* **103**, 474-481.
- Stanton, M. E., Mundy, W. R., Ward, T., Dulchinos, V., and Barry, C. C. (1994). Time-dependent effects of acute chlorpyrifos administration on spatial delayed alternation and cholinergic neurochemistry in weanling rats. *Neurotoxicology* **15**, 201-208.
- Undem, B. J., and Carr, M. J. (2002). The role of nerves in asthma. *Curr. Allergy Asthma Rep.* **2**, 159-165.
- USDA (2003). Agricultural chemicals and production technology, Vol. 2003. Economic Research Service.
- U.S. EPA (2000). Overview of Diazinon Revised Risk Assessment, 12/05/00. U.S. Environmental Protection Agency. <http://www.epa.gov/pesticides/op/diazinon/overview.pdf>.
- Wagner, E. M., and Jacoby, D. B. (1999). Methacholine causes reflex bronchoconstriction. *J. Appl. Physiol.* **86**, 294-297.
- Ward, T. R., and Mundy, W. R. (1996). Organophosphorus compounds preferentially affect second messenger systems coupled to M2/M4 receptors in rat frontal cortex. *Brain Res. Bull.* **39**, 49-55.
- Weisenburger, D. D. (1993). Human health effects of agricultural use. *Hum. Pathol.* **24**, 571-576.
- Weitzman, M., Gortmaker, S. L., Sobol, A. M., and Perrin, J. M. (1992). Recent trends in the prevalence and severity of childhood asthma. *JAMA* **268**, 2673-2677.
- Whitmore, R. W., Kelly, J. E., and Reading, P. L. (2003). National Homes and Garden Pesticide Survey: Final Report, Vol 1. Research Triangle Park, NC: Research Triangle Institute, Research Triangle Park, NC.
- Whyatt, R. M., Camann, D. E., Kinney, P. L., Reyes, A., Ramirez, J., Dietrich, J., Diaz, D., Holmes, D., and Perera, F. P. (2002). Residential pesticide use during pregnancy among a cohort of urban minority women. *Environ. Health Perspect.* **110**, 507-514.
- Wilhoit, L., Davidson, N., Supkoff, D., Steggall, J., Braun, A., Simmons, S., Hobza, B., Hunter, C., Gorder, N., Goodman, C., et al. (1999). Pesticide Use Analysis and Trends from 1991 to 1996. State of California Environmental Protection Agency, Sacramento, CA.
- Zhang, W., Basile, A. S., Gomeza, J., Volpicelli, L. A., Levey, A. I., and Wess, J. (2002). Characterization of central inhibitory muscarinic autoreceptors by the use of muscarinic acetylcholine receptor knock-out mice. *J. Neurosci.* **22**, 1709-1717.
- Zhu, S. Z., Wang, S. Z., Abdallah, E. A., and el-Fakahany, E. E. (1991). DFP-induced regulation of cardiac muscarinic receptor mRNA *in vivo* measured by DNA-excess solution hybridization. *Life Sci.* **48**, 2579-2584.

## Mechanisms of organophosphate insecticide-induced airway hyperreactivity

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**Fryer, Allison D., Pamela J. Lein, Angela S. Howard, Bethany L. Yost, Rondell A. Beckles, and David A. Jett.** Mechanisms of organophosphate insecticide-induced airway hyperreactivity. *Am J Physiol Lung Cell Mol Physiol* 286: L963–L969, 2004. First published January 2, 2004; 10.1152/ajplung.00343.2003.—It has been suggested that pesticide exposure may be a contributing factor underlying the increased incidence of asthma in the United States and other industrialized nations. To test this hypothesis, airway hyperreactivity was measured in guinea pigs exposed to chlorpyrifos, a widely used organophosphate pesticide. Electrical stimulation of the vagus nerves caused frequency-dependent bronchoconstriction that was significantly potentiated in animals 24 h or 7 days after a single subcutaneous injection of either 390 mg/kg or 70 mg/kg of chlorpyrifos, respectively. Mechanisms by which chlorpyrifos may cause airway hyperreactivity include inhibition of acetylcholinesterase (AChE) or dysfunction of M3 muscarinic receptors on airway smooth muscle or of autoinhibitory M2 muscarinic receptors on parasympathetic nerves in the lung. AChE activity in the lung was significantly inhibited 24 h after treatment with 390 mg/kg of chlorpyrifos, but not 7 days after injection of 70 mg/kg of chlorpyrifos. Acute exposure to eserine (250 µg/ml) also significantly inhibited lung AChE but did not potentiate vagally induced bronchoconstriction. Neuronal M2 receptor function was tested using the M2 agonist pilocarpine, which inhibits vagally induced bronchoconstriction in control animals. In chlorpyrifos-treated animals, pilocarpine dose-response curves were shifted significantly to the right, demonstrating decreased responsiveness of neuronal M2 receptors. In contrast, chlorpyrifos treatment did not alter methacholine-induced bronchoconstriction, suggesting that chlorpyrifos does not alter M3 muscarinic receptor function on airway smooth muscle. These data demonstrate that organophosphate insecticides can cause airway hyperreactivity in the absence of AChE inhibition by decreasing neuronal M2 receptor function.

asthma; pesticide; muscarinic receptor; cholinesterase

OVER THE PAST 20 YEARS there has been a significant increase in the incidence of asthma in industrialized nations, particularly in children in urban settings (33). Over this same period, the use of insecticides, particularly organophosphate insecticides, has increased significantly not only in agricultural (26, 45, 71, 75) but also residential and urban settings (6, 15, 50, 73, 74). A number of clinical and epidemiological studies have linked exposure to organophosphates to airway hyperreactivity and other symptoms of asthma (12, 19, 35, 59). However, the association between organophosphates and asthma has not been tested in an experimental model system and the mechanism(s) by which this class of insecticides causes changes in airway function remains speculative.

In the lung, cholinergic nerves in the vagi mediate airway tone and reactivity. These nerves release acetylcholine onto M3 muscarinic receptors causing contraction of airway smooth muscle, resulting in bronchoconstriction. Vagally induced bronchoconstriction is limited by autoinhibitory M2 muscarinic receptors on parasympathetic nerves (28, 54). Previous studies have shown that neuronal M2 receptors are dysfunctional in animal models of asthma (29, 31, 37) and in patients with asthma (55). Loss of M2 receptor function leads to increased release of acetylcholine from the parasympathetic nerves resulting in potentiation of vagally mediated bronchoconstriction, which contributes to airway hyperreactivity.

Organophosphates are known to alter cholinergic function in the brain (53, 60). The generally accepted mechanism of organophosphate neurotoxicity following acute exposure to high doses is inhibition of acetylcholinesterase (AChE). It has been proposed that this same mechanism underlies the effects of organophosphate insecticides on bronchoconstriction (14, 67). However, there is evidence that in the brain, low-level doses of organophosphate pesticides that do not inhibit AChE may alter cholinergic neurotransmission via direct effects on muscarinic and nicotinic receptor function (1, 7, 36, 39, 40, 42–44). These observations led us to test whether chlorpyrifos (Dursban, Lorsban), a commonly used organophosphate with documented widespread human exposure (15, 17, 25, 34, 48), potentiates vagally induced bronchoconstriction by increasing cholinergic drive in the lungs via inhibition of AChE and/or alteration of neuronal M2 or airway smooth muscle M3 muscarinic receptor function.

### METHODS

**Animals.** Specific pathogen-free male guinea pigs (300–350 g) were shipped from Hilltop Lab Animals (Scottsdale, PA) in filtered crates, housed in high-efficiency, particulate-filtered air, and fed a normal diet (Prolab; Agway, Syracuse, NY). All protocols were approved by The Johns Hopkins University Animal Care and Use Committee.

**Chlorpyrifos and eserine exposure.** Chlorpyrifos (*o,o*-diethyl *o*-[3,5,6-trichloro-2-pyridinol] phosphorothionate, 99.5% pure) was purchased from Chem Service (West Chester, PA) and used within 1 mo of purchase with interim storage as recommended by the manufacturer. Chlorpyrifos dissolved in peanut oil at 70 mg/kg or 390 mg/kg or an equal volume (300 µl) of peanut oil alone was administered to guinea pigs by subcutaneous injection in the subscapular region. Although the most significant routes of chlorpyrifos exposure in humans are oral ingestion and inhalation (30), subcutaneous dosing is commonly used in mechanistic studies of chlorpyrifos and other organophosphates (13, 16, 62, 70). Subcutaneous administration of

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chlorpyrifos allows gradual release into the systemic circulation (63), which closely resembles human exposures (30). The guinea pig throat anatomy occludes a stomach tube more so than with rats or mice. Thus the subcutaneous method is also much less stressful to a guinea pig relative to gavage. Animals dosed with chlorpyrifos were monitored for signs of cholinergic intoxication (tremors, altered gait, and excessive excretions) at 1 and 24 h following injections. Guinea pigs given 70 mg/kg sc were tested for airway hyperreactivity 7 days postinjection, whereas animals receiving 390 mg/kg sc were tested 24 h later. These dosing paradigms were found to inhibit lung AChE activity by 0 and 50%, respectively (see Fig. 1), approximating the organophosphate exposures typically observed in humans, which include chronic exposure to low doses that have negligible effect on AChE or acute/subacute exposure to doses that significantly inhibit AChE (30). A 50% inhibition of AChE was chosen as a target value since this level of AChE inhibition is not uncommon in acutely exposed humans (30), and overt toxicity to chlorpyrifos in rodents becomes manifest when brain AChE is inhibited by >60% (57). Vehicle controls treated with peanut oil subcutaneously were tested 7 days later, and since the physiological values obtained with these animals did not differ from those measured in animals that were tested 24 h after saline injections (see Fig. 2), peanut oil controls were not performed for the cohort of animals tested 24 h after chlorpyrifos injection. Eserine was dissolved in sterile saline and administered at a dosage of 0.25 mg/kg iv to anesthetized animals 15 min before physiological measurements, as described below.

**Anesthesia and measurement of pulmonary inflation pressure.** Guinea pigs were anesthetized (1.5 g/kg urethane ip), and blood pressure and heart rate were measured from the carotid artery. Both jugular veins were cannulated for administration of drugs. Both vagus nerves were cut and placed on electrodes under oil. Succinylcholine (10  $\mu$ g/kg iv) was infused to paralyze the animals, and they were ventilated via a tracheal cannula with a positive pressure constant volume (1 ml/100 g body wt and 100 breaths/min). Pulmonary inflation pressure ( $P_{pi}$ ) was measured via a side arm at the trachea; bronchoconstriction was measured as the increase in  $P_{pi}$  over the pressure produced by the ventilator as previously described (28, 29, 37).

**Measurement of vagally induced bronchoconstriction.** Noradrenaline was depleted 25 min before the start of the experiment with guanethidine (10 mg/kg iv). Both vagi were stimulated (0.2 ms, 10 V,

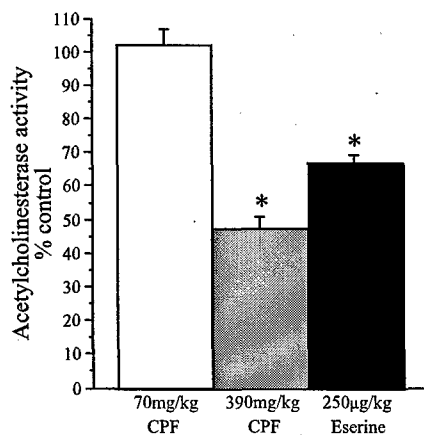


Fig. 1. Mean acetylcholinesterase (AChE) levels in the lungs of guinea pigs treated with vehicle were  $3.8 \pm 0.15$   $\mu$ mol substrate $\cdot$ min $^{-1}$  $\cdot$ mg $^{-1}$  protein. Treatment of animals with a single dose of 70 mg/kg of chlorpyrifos (CPF) did not inhibit AChE activity in the lungs 7 days postinjection (open bar). In contrast, 390 mg/kg of chlorpyrifos after 24 h (gray bar) and 250  $\mu$ g/kg of eserine (solid bar) significantly inhibited AChE activity in the lungs. Each point is the mean  $\pm$  SE of 5–8 animals presented as a % of control. \*Significantly different from control.

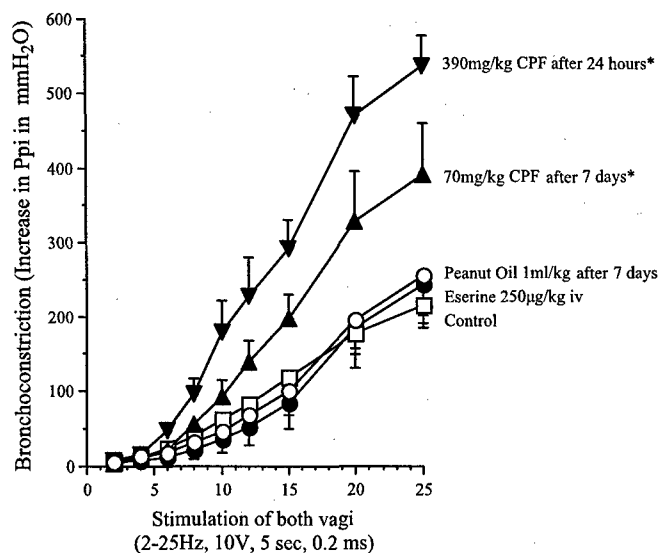


Fig. 2. Electrical stimulation of the vagus nerves (1–25 Hz, 10 V, 0.2 ms, 5-s train) produced frequency-dependent bronchoconstriction in the lungs, measured as an increase in pulmonary inflation pressure ( $P_{pi}$ ;  $\square$ ). Vagally induced bronchoconstriction was significantly increased in animals 7 days after a single dose of 70 mg/kg of chlorpyrifos ( $\blacktriangle$ ) and 24 h after a dose of 390 mg/kg of chlorpyrifos ( $\blacktriangledown$ ). Frequency response curves in animals 7 days after treatment once with peanut oil vehicle ( $\circ$ ) or acutely with 250  $\mu$ g/kg of eserine (iv;  $\bullet$ ), a nonorganophosphate AChE inhibitor, were not different from control animals. Each point is the mean  $\pm$  SE of 5–8 animals. \*Significantly different from control.

1–25 Hz, 5-s duration at 2-min intervals) producing frequency-dependent bronchoconstriction and bradycardia due to release of acetylcholine onto postjunctional M2 muscarinic receptors in the heart and postjunctional M3 muscarinic receptors in the lungs. Both vagally induced bronchoconstriction and bradycardia could be abolished by atropine (1 mg/kg iv).

**Measurement of neuronal M2 and smooth muscle M3 muscarinic receptor function.** The function of neuronal M2 receptors was determined using a standard assay (28, 29, 37): the ability of the muscarinic agonist pilocarpine to inhibit the bronchoconstrictor response to vagal stimulation at 2 Hz. Pilocarpine inhibits vagally induced bronchoconstriction via stimulation of the neuronal M2 receptors at doses that are 100-fold less than the doses required to cause bronchoconstriction by stimulating postjunctional M3 receptors (28). The effect of pilocarpine on vagally induced bronchoconstriction is reported as the ratio of bronchoconstriction in the presence of pilocarpine to bronchoconstriction in the absence of pilocarpine. Decreased inhibition by pilocarpine of vagally induced bronchoconstriction, manifested as a shift to the right of the dose-response curve, indicates M2 receptor dysfunction (28, 29, 37).

In vagotomized guinea pigs, parasympathetic nerves that release acetylcholine and contain M2 receptor are absent. To assess the effects of organophosphates on M3 muscarinic receptor function in airway smooth muscle and to bypass endogenous acetylcholine release from parasympathetic nerves, we measured bronchoconstriction in vagotomized guinea pigs in response to methacholine (1–10  $\mu$ g/kg iv), which is not readily metabolized by AChE (11, 56), and acetylcholine (1–8  $\mu$ g/kg iv). The dose-response curve of bronchoconstriction in response to acetylcholine was compared with that of methacholine as well as with vagally induced bronchoconstriction to determine whether changes in vagally induced bronchoconstriction occur via changes in the nerves, in AChE activity, or postjunctional M3 receptor function.

**AChE assay.** Immediately after the completion of physiological measurements, lungs, brain, and heparinized blood samples were

obtained for determination of AChE activity via the standard Ellman assay (23) using DTNB and acetylthiocholine iodide (ASChI) as the substrate. Assays were run against blanks containing DTNB. The reaction was started with the addition of ASChI after equilibration for 2–3 min. Hydrolysis of ASChI was determined by monitoring the change in absorbance at 405 nm. To inhibit pseudocholinesterase activity, 100  $\mu$ M tetraisopropyl pyrophosphoramidate was included in the assay. Data from lung and brain samples were normalized using protein concentration determined using the bicinchoninic assay according to the manufacturer's directions (Pierce, Rockford, IL). AChE activity in blood samples was normalized according to the number of red blood cells as determined using a hemacytometer.

**Statistics.** Data are expressed as means  $\pm$  SE. Frequency, pilocarpine, methacholine, and acetylcholine dose-response curves were analyzed using a two-way analysis of variance for repeated measures. Baseline heart rates (beats/minute), blood pressures (mmHg),  $P_{pi}$  (mmH<sub>2</sub>O), and changes in  $P_{pi}$  (mmH<sub>2</sub>O before pilocarpine administration) as well as AChE activity levels (as % of control) were analyzed using ANOVA (Statview 4.5; Abacus Concepts, Berkeley, CA). A *P* value of 0.05 was considered significant.

## RESULTS

Treatment with chlorpyrifos did not result in any apparent signs of cholinergic intoxication in guinea pigs 1 or 24 h after subcutaneous injections of 70 or 390 mg/kg. AChE activity in the lungs was not altered 7 days after a single injection of 70 mg/kg of chlorpyrifos (Fig. 1). However, 24 h after a single injection of 390 mg/kg of chlorpyrifos, lung AChE was significantly inhibited by 50%. Acute exposure to 250  $\mu$ g/kg of eserine, a nonorganophosphate anticholinesterase, caused a level of AChE inhibition that was not significantly different from that observed with the higher dose of chlorpyrifos (Fig. 1).

Neither chlorpyrifos nor peanut oil altered baseline  $P_{pi}$  (control  $91 \pm 7$  mmH<sub>2</sub>O, peanut oil  $96 \pm 5$  mmH<sub>2</sub>O, 70 mg/kg of chlorpyrifos  $87 \pm 7$  mmH<sub>2</sub>O, 390 mg/kg of chlorpyrifos  $96 \pm 5$  mmH<sub>2</sub>O), resting heart rate (control  $281 \pm 11$  beats/min, peanut oil  $271 \pm 8$  beats/min, 70 mg/kg of chlorpyrifos  $268 \pm 9$  beats/min, 390 mg/kg of chlorpyrifos  $300 \pm 7$  beats/min), or resting diastolic blood pressure (control  $45 \pm 2.3$  mmHg, peanut oil  $41 \pm 2$  mmHg, 70 mg/kg of chlorpyrifos  $46 \pm 2$  mmHg, 390 mg/kg of chlorpyrifos  $56 \pm 2.4$  mmHg) in vagotomized, anesthetized guinea pigs.

Electrical stimulation of both vagi (1–25 Hz) caused a frequency-dependent increase in bronchoconstriction that was significantly potentiated in animals 24 h after a single injection of 390 mg/kg of chlorpyrifos or 7 days after a single injection of 70 mg/kg of chlorpyrifos (Fig. 2). However, a greater increase in bronchoconstriction was observed in animals that received the acute high-dose chlorpyrifos treatment. In contrast, eserine, which significantly inhibited AChE (Fig. 1), did not potentiate vagally induced bronchoconstriction. Vagally induced bronchoconstriction was not altered in animals receiving vehicle alone (peanut oil) relative to control animals.

Neuronal M2 receptor function was tested in chlorpyrifos-treated animals using the muscarinic agonist pilocarpine. Before pilocarpine was administered, simultaneous electrical stimulation of both vagus nerves (2 Hz, 0.2 ms, 5–20 V, 22 s at 1-min intervals) produced transient bronchoconstriction (measured as an increase in  $P_{pi}$ ) that did not differ among groups (control  $27.6 \pm 0.2$  mmH<sub>2</sub>O, peanut oil  $19.8 \pm 3$  mmH<sub>2</sub>O, 70 mg/kg of chlorpyrifos  $18.6 \pm 5$  mmH<sub>2</sub>O, 390

mg/kg of chlorpyrifos  $24.6 \pm 6$  mmH<sub>2</sub>O). In guinea pigs treated with peanut oil, pilocarpine (1–100  $\mu$ g/kg iv) dose dependently inhibited vagally induced bronchoconstriction, demonstrating that the neuronal M2 receptors are functional (Fig. 3,  $\circ$ ). The effect was identical to saline-treated controls (not shown), demonstrating that injection of peanut oil subcutaneously for 7 days did not alter the function of neuronal M2 receptors. The dose-response curve to pilocarpine was shifted significantly to the right in animals 24 h after treatment with chlorpyrifos at 390 mg/kg. A lesser, but still significant, rightward shift was observed 7 days after treatment with 70 mg/kg of chlorpyrifos. Shifting of the pilocarpine dose-response curve to the right is consistent with decreased function of the M2 receptors.

To test the direct response of M3 receptors on airway smooth muscle to muscarinic agonists, bronchoconstriction induced by intravenous methacholine, which is not rapidly metabolized by AChE, and by intravenous acetylcholine was measured in vagotomized guinea pigs. Chlorpyrifos treatment did not alter methacholine-induced bronchoconstriction (Fig. 4A). Because M2 receptors on vagus nerves were not present in these vagotomized guinea pigs, the absence of an effect on methacholine-induced bronchoconstriction indicates that chlorpyrifos does not affect the ability of agonists to interact with postjunctional M3 muscarinic receptors. Acetylcholine-induced bronchoconstriction was significantly increased in animals treated with 390 mg/kg of chlorpyrifos (Fig. 4B). Eserine at a dosage of 250  $\mu$ g/kg also potentiated acetylcholine-induced bronchoconstriction, and although the eserine dose response did not differ significantly from the control dose-response curve, it also did not differ significantly from the dose-response curve obtained from animals treated with 390 mg/kg of chlorpyrifos. Thus eserine potentiated bronchoconstriction to an intermediate level between that of control

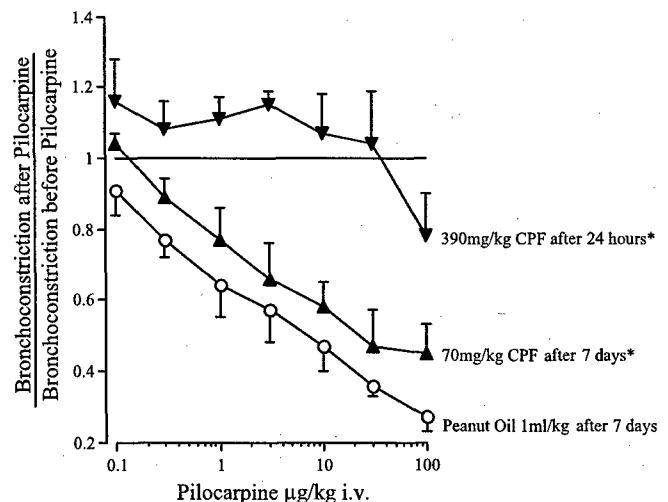


Fig. 3. Neuronal M2 receptor function was tested using pilocarpine. Increasing doses of pilocarpine inhibited vagally induced bronchoconstriction in a dose-related manner in animals 7 days after treatment with peanut oil vehicle ( $\circ$ ), demonstrating the presence of functional M2 receptors. The effect of pilocarpine was shifted significantly to the right in animals 7 days after treatment with 70 mg/kg of chlorpyrifos ( $\blacktriangle$ ). In the animals treated with 390 mg/kg of chlorpyrifos ( $\blacktriangledown$ ), pilocarpine did not inhibit vagally induced bronchoconstriction, indicating neuronal M2 muscarinic receptor dysfunction after 24 h. Each point is the mean  $\pm$  SE of 5 animals. \*Significantly different from control.

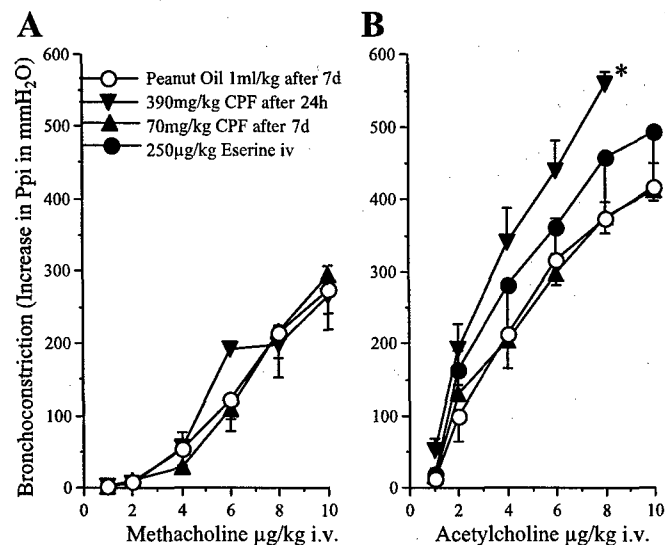


Fig. 4. *A*: methacholine (1–10 µg/kg iv)-induced bronchoconstriction in vagotomized guinea pigs was not different between peanut oil (○)- or chlorpyrifos-treated guinea pigs (7 days after 70 mg/kg of chlorpyrifos, ▲; and 24 h after 390 mg/kg of chlorpyrifos, ▼). *B*: acetylcholine (1–10 µg/kg iv)-induced bronchoconstriction in vagotomized guinea pigs was significantly potentiated by 390 mg/kg of chlorpyrifos, whereas 70 mg/kg of chlorpyrifos had no effect. Acute treatment with 250 µg/kg of eserine (iv, ●) caused a small but not statistically significant potentiation of acetylcholine-induced bronchoconstriction. Each point is the mean  $\pm$  SE of 5 animals. \*Significantly different from control; d, days.

animals and animals treated with 390 mg/kg of chlorpyrifos. Treatment of animals with 70 mg/kg of chlorpyrifos for 7 days had no effect on acetylcholine-induced bronchoconstriction. The potentiation of acetylcholine-induced bronchoconstriction by the higher dose of chlorpyrifos and by eserine is consistent with the inhibition of AChE by these treatments (see Fig. 1).

M2 muscarinic receptors are also present in the heart. Thus we measured vagally induced bradycardia to determine whether organophosphate insecticides alter M2 muscarinic receptors in tissues other than the lung. In the heart, stimulation of the vagus nerves (1–25 Hz) produces bradycardia that is frequency dependent (Fig. 5). Treatment with 70 mg/kg of chlorpyrifos did not alter the vagally induced fall in heart rate relative to control. However, in the animals treated with 390 mg/kg of chlorpyrifos for 24 h, the frequency response curve was potentiated at frequencies <20 Hz (Fig. 5). At 20 and 25 Hz, the fall in heart rate approaches maximum, and the differences are no longer significant. However, the potentiation of vagally induced bradycardia by the higher dose of chlorpyrifos does not appear to be mediated by inhibition of AChE since eserine at a concentration that significantly inhibits AChE does not potentiate vagally induced bradycardia (Fig. 5). Methacholine- and acetylcholine-induced bradycardia was not altered by either high- or low-dose chlorpyrifos treatment (Fig. 6) despite inhibition of AChE by the higher dose of chlorpyrifos.

## DISCUSSION

In humans, exposure to organophosphate insecticides and other pesticides has been associated with a variety of respiratory symptoms, including decreased forced expiratory volume in 1 min, wheeze, cough, and shortness of breath (3, 19, 35, 46, 47, 65, 67). Similarly, it has been reported that organophos-

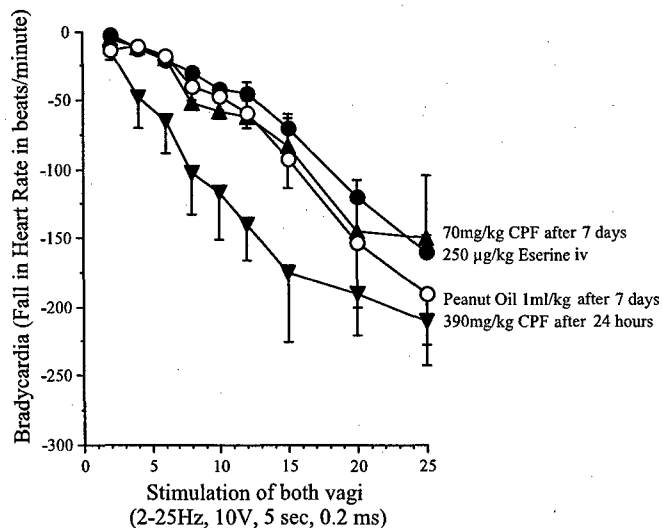


Fig. 5. Electrical stimulation of the vagus nerves (1–25 Hz, 10 V, 0.2 ms, 5-s train) produced frequency-dependent bradycardia in the heart measured as a fall in heart rate (○). Vagally induced bradycardia in animals 7 days after treatment with 70 mg/kg of chlorpyrifos (▲) or acutely with 250 µg/kg of eserine (●) was not significantly different from peanut oil-treated animals. However, vagally induced bradycardia in animals treated with 390 mg/kg of chlorpyrifos after 24 h (▼) was shifted to the left. Up until 20 Hz, the shift was significantly different from oil control; however, at 20 and 25 Hz, the fall in heart rate was approaching maximum, and the differences were no longer significant. Each point is the mean  $\pm$  SE of 5–8 animals.

phate insecticides induce bronchospasm in a variety of animals (20, 32, 66). Our data provide more direct evidence of a causal link between organophosphate exposure and airway hyperactivity. Specifically, we observed that vagally induced bronchoconstriction in guinea pig lungs is significantly potentiated

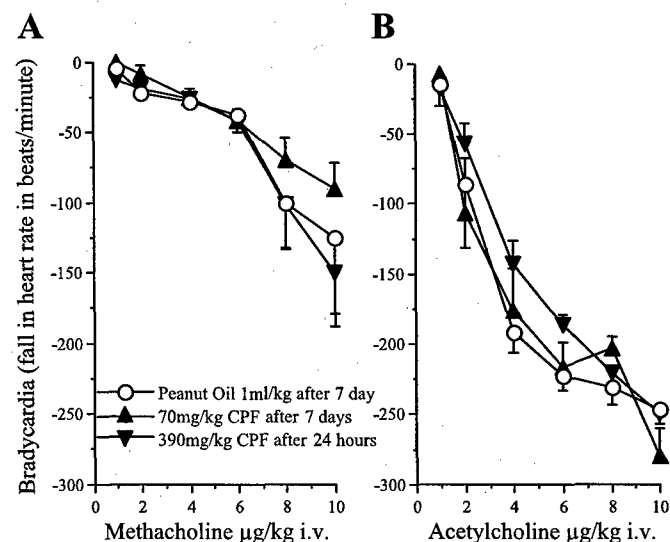


Fig. 6. *A*: methacholine (1–10 µg/kg iv)-induced bradycardia in vagotomized guinea pigs was not different between peanut oil (○)- or chlorpyrifos-treated guinea pigs (7 days after 70 mg/kg of chlorpyrifos, ▲; and 24 h after 390 mg/kg of chlorpyrifos, ▼). *B*: acetylcholine (1–10 µg/kg iv)-induced bradycardia in vagotomized guinea pigs was not different between peanut oil (○)- or chlorpyrifos-treated guinea pigs (7 days after 70 mg/kg of chlorpyrifos, ▲; and 24 h after 390 mg/kg of chlorpyrifos, ▼). Each point is the mean  $\pm$  SE of 5 animals.

following either acute (24 h after 390 mg/kg of chlorpyrifos) or chronic (7 days after 70 mg/kg) exposure to chlorpyrifos.

It has been proposed that if organophosphates contribute to asthma, it is likely due to inhibition of AChE (14, 24, 67), which is the principal mechanism underlying acute organophosphate neurotoxicity. Support for this hypothesis includes observations that not only organophosphate insecticides, but also other structurally unrelated AChE-inhibiting insecticides, such as carbaryl, enhance airway hyperreactivity in rats (20) and humans (67). However, two observations from our studies suggest mechanisms other than AChE inhibition mediate chlorpyrifos effects on vagally induced bronchoconstriction. First, our data indicate that animals tested 7 days after receiving 70 mg/kg of chlorpyrifos (subcutaneously) exhibit airway hyperreactivity in the absence of AChE inhibition. Second, acute administration of the nonorganophosphate eserine at a dose (250  $\mu$ g/ml) that significantly inhibits AChE does not potentiate vagally induced bronchoconstriction. Similarly, a poor correlation has been noted between cholinesterase inhibition and toxic effects in the brain by some organophosphate insecticides (22), prompting investigations of alternative mechanisms of neurotoxicity. These observations are important because they suggest that toxicity from some organophosphates may occur below thresholds of exposure normally defined by AChE inhibition.

Neuronal M2 muscarinic receptors limit release of acetylcholine from the vagus nerves in the lungs (28). Pharmacological blockade of neuronal M2 receptors increases release of acetylcholine from the nerves (5, 27), which potentiates vagally induced bronchoconstriction (28, 29, 37). Our data show that neuronal M2 receptor function is inhibited by both high and low doses of chlorpyrifos, consistent with other findings that organophosphate insecticides act on muscarinic receptors in the brain (1, 36, 38, 43). This effect of chlorpyrifos on vagally induced bronchoconstriction is dependent on the dosing regimen. Vagally induced bronchoconstriction was significantly greater in animals treated with the high dose of chlorpyrifos relative to animals treated with the low dose (Fig. 2). A similar dependency was observed for the effects of chlorpyrifos on M2 receptor function as determined by pilocarpine dose-response curves (Fig. 3). In contrast, neither dose of chlorpyrifos changed the response to intravenous methacholine, demonstrating that the function of M3 muscarinic receptors on airway smooth muscle was not altered in animals in which M2 receptors mediating ACh release were not present. Selective loss of neuronal M2 receptor function in the lungs is also associated with other models of airway hyperreactivity, including antigen challenge (29), viral infection (37), and exposure to ozone (31), suggesting that decreased M2 receptor function on airway nerves is a generalized mechanism underlying airway hyperreactivity.

The ability of organophosphate insecticides to inhibit neuronal M2 receptors may not be restricted to the lungs. Organophosphate insecticides have been shown to inhibit muscarinic receptor binding in the brain (1, 7, 36, 39, 40, 43, 44) and bind to a subpopulation of M2 receptors in the heart (68). In the heart, M2 receptors are present on parasympathetic nerves that supply the heart where they function to inhibit release of acetylcholine (52, 58) as well as on cardiac muscle where they mediate bradycardia (10, 51). The single high dose of chlorpyrifos potentiated vagally induced bradycardia but not brady-

cardia induced by intravenous administration of acetylcholine. This is consistent with loss of neuronal M2 receptor function on parasympathetic nerves in the heart resulting in increased release of acetylcholine, which potentiates vagally induced bradycardia. Neither dose of chlorpyrifos altered bradycardia induced by acetylcholine or methacholine administered intravenously, indicating that the postjunctional M2 receptors are not sensitive to organophosphate insecticides, and this is consistent with the sensitivity of neuronal M2 receptors we observed in the lung. These data are also consistent with previous observations that the function of the neuronal M2 receptors in the heart are inhibited by systemic administration of double-stranded RNA, which does not alter the function of postjunctional M2 receptors (9). Thus the neuronal receptors appear to be more vulnerable to inhibition than the postjunctional receptors.

The mechanism(s) by which organophosphate insecticides alter M2 receptor function in the lungs have yet to be elucidated. Mechanisms by which these compounds alter muscarinic receptor function in neurons include downregulation of muscarinic receptor expression (39, 40), modulation of ligand binding to muscarinic receptors (38, 43, 44), and alteration of signal transduction pathways downstream of muscarinic receptor activation (8, 36, 72). In vitro studies of cardiac M2 muscarinic receptors have demonstrated that acute exposure to the oxon metabolite of chlorpyrifos alters ligand binding via diethylphosphorylation of the receptor itself (7). Whether these mechanisms underlie the effects of organophosphates on neuronal M2 receptor function in the lung has yet to be determined.

Data presented here indicate that organophosphate insecticides potentiate vagally induced bronchoconstriction via disruption of the cholinergic control of airway responsiveness. A significant finding from our studies is that chlorpyrifos altered neuronal M2 receptor function in the lung at concentrations that did not inhibit AChE. Although the threshold concentration for this effect was not determined in our studies, it has been shown that ligand binding to muscarinic receptors in the brain (43) as well as signaling pathways downstream of muscarinic receptor binding (64) can be disrupted by very low (nanomolar to picomolar) concentrations of organophosphate insecticides. These data suggest that exposure to not only occupational, but also environmental, levels of these compounds may have biological consequences. The significance of these findings to public health is heightened by evidence of widespread human exposure to chlorpyrifos and other organophosphate insecticides (34, 45).

Children represent a potentially sensitive subpopulation with respect to asthma. Thus it is of great concern that in a sample of 84,000 children across the United States, the urinary levels of chlorpyrifos metabolites were above the detection limit 98% of the time, compared with a 4% detection rate for the herbicide atrazine (2). Many of the organophosphate insecticides have been restricted or banned due to their developmental neurotoxicity in animals. However, many of these compounds, including chlorpyrifos, are still used commercially in both agricultural settings and urban environments. These pesticide usage patterns correlate positively with reports of high incidence of asthma morbidity in agricultural workers (3, 35, 41, 46, 67, 69, 76) and in residents of the inner cities of the United States (18, 33, 49, 61). The coincident rise in the incidence of

asthma (33) and in the use of organophosphate insecticides (26, 45, 71, 75) suggests a potential causal relationship between these two observations, which is corroborated by our data obtained using an animal model of airway hyperreactivity. Our data raise significant questions regarding the current use of organophosphate insecticides in the inner cities to control cockroach antigen (6, 15, 50, 73, 74), which itself has been associated with asthma (4, 21), and suggest that exposure to insecticides may be contributing to rather than ameliorating asthma.

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#### REFERENCES

- Abdallah EA, Jett DA, Eldefrawi ME, and Eldefrawi AT. Differential effects of paraoxon on the M3 muscarinic receptor and its effector system in rat submaxillary gland cells. *J Biochem Toxicol* 7: 125-132, 1992.
- Adgate JL, Barr DB, Clayton CA, Eberly LE, Freeman NC, Liroy PJ, Needham LL, Pellizzari ED, Quackenboss JJ, Roy A, and Sexton K. Measurement of children's exposure to pesticides: analysis of urinary metabolite levels in a probability-based sample. *Environ Health Perspect* 109: 583-590, 2001.
- Arif AA, Whitehead LW, Delclos GL, Tortolero SR, and Lee ES. Prevalence and risk factors of work related asthma by industry among United States workers: data from the third national health and nutrition examination survey (1988-1994). *Occup Environ Med* 59: 505-511, 2002.
- Arruda LK, Vailes LD, Ferriani VP, Santos AB, Pomes A, and Chapman MD. Cockroach allergens and asthma. *J Allergy Clin Immunol* 107: 419-428, 2001.
- Baker DG, Don HF, and Brown JK. Direct measurement of acetylcholine release in guinea pig trachea. *Am J Physiol Lung Cell Mol Physiol* 263: L142-L147, 1992.
- Berkowitz GS, Obel J, Deych E, Lapinski R, Godbold J, Liu Z, Landrigan PJ, and Wolff MS. Exposure to indoor pesticides during pregnancy in a multiethnic, urban cohort. *Environ Health Perspect* 111: 79-84, 2003.
- Bomser JA and Casida JE. Diethylphosphorylation of rat cardiac M2 muscarinic receptor by chlorpyrifos oxon in vitro. *Toxicol Lett* 119: 21-26, 2001.
- Bomser JA, Quistad GB, and Casida JE. Chlorpyrifos oxon potentiates diacylglycerol-induced extracellular signal-regulated kinase (ERK 44/42) activation, possibly by diacylglycerol lipase inhibition. *Toxicol Appl Pharmacol* 178: 29-36, 2002.
- Bowerfind WM, Fryer AD, and Jacoby DB. Double-stranded RNA causes airway hyperreactivity and neuronal M2 muscarinic receptor dysfunction. *J Appl Physiol* 92: 1417-1422, 2002.
- Brodde OE, Bruck H, Leineweber K, and Seyfarth T. Presence, distribution and physiological function of adrenergic and muscarinic receptor subtypes in the human heart. *Basic Res Cardiol* 96: 528-538, 2001.
- Bruning TA, Chang PC, Kemme MJ, Vermeij P, Pffaffendorf M, and van Zwieten PA. Comparison of cholinergic vasodilator responses to acetylcholine and methacholine in the human forearm. *Blood Press* 5: 333-341, 1996.
- Bryant DH. Asthma due to insecticide sensitivity. *Aust NZ J Med* 15: 66-68, 1985.
- Bushnell PJ, Padilla SS, Ward T, Pope CN, and Olszyk VB. Behavioral and neurochemical changes in rats dosed repeatedly with diisopropylfluorophosphate. *J Pharmacol Exp Ther* 256: 741-750, 1991.
- Casarett LJ and Doull J. *Toxicology*. New York: Macmillan, 1975.
- CDC. *Second National Report on Human Exposure to Environmental Chemicals*. Atlanta, GA: National Center for Environmental Health, 2003.
- Chiappa S, Padilla S, Koenigsberger C, Moser V, and Brimijoin S. Slow accumulation of acetylcholinesterase in rat brain during enzyme inhibition by repeated dosing with chlorpyrifos. *Biochem Pharmacol* 49: 955-963, 1995.
- Davis DL and Ahmed AK. Exposures from indoor spraying of chlorpyrifos pose greater health risks to children than currently estimated. *Environ Health Perspect* 106: 299-301, 1998.
- Delfino RJ, Gong H Jr, Linn WS, Pellizzari ED, and Hu Y. Asthma symptoms in Hispanic children and daily ambient exposures to toxic and criteria air pollutants. *Environ Health Perspect* 111: 647-656, 2003.
- Deschamps D, Questel F, Baud FJ, Gervais P, and Dally S. Persistent asthma after acute inhalation of organophosphate insecticide. *Lancet* 344: 1712, 1994.
- Dong W, Gilmour MI, Lambert AL, and Selgrade MK. Enhanced allergic responses to house dust mite by oral exposure to carbaryl in rats. *Toxicol Sci* 44: 63-69, 1998.
- Eggleston PA and Arruda LK. Ecology and elimination of cockroaches and allergens in the home. *J Allergy Clin Immunol* 107: S422-S429, 2001.
- Eldefrawi AT, Jett DA, and Eldefrawi ME. Direct actions of organophosphate anticholinesterases on muscarinic receptors. In: *Organophosphates: Chemistry, Fate and Effects*, edited by Chambers JE and Levi PE. New York: Academic, 1992, p. 419-432.
- Ellman GL, Courtney KD, Andres V, and Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7: 88-95, 1961.
- Ernst P. Pesticide exposure and asthma. *Am J Respir Crit Care Med* 165: 563-564, 2002.
- Eskenazi B, Bradman A, and Castorina R. Exposures of children to organophosphate pesticides and their potential adverse health effects. *Environ Health Perspect* 107, Suppl 3: 409-419, 1999.
- Fenske RA, Lu C, Barr D, and Needham L. Children's exposure to chlorpyrifos and parathion in an agricultural community in central Washington State. *Environ Health Perspect* 110: 549-553, 2002.
- Fryer AD, Elbon CL, Kim AL, Xiao HQ, Levey AI, and Jacoby DB. Cultures of airway parasympathetic nerves express functional M2 muscarinic receptors. *Am J Respir Cell Mol Biol* 15: 716-725, 1996.
- Fryer AD and MacLagan J. Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br J Pharmacol* 83: 973-978, 1984.
- Fryer AD and Wills-Karp M. Dysfunction of M2-muscarinic receptors in pulmonary parasympathetic nerves after antigen challenge. *J Appl Physiol* 71: 2255-2261, 1991.
- Gallo MA and Lawryk NJ. Organic phosphorus pesticides. In: *Handbook of Pesticide Toxicology*, edited by Hayes WJ and Laws ER. New York: Academic, 1991, p. 917-972.
- Gambone LM, Elbon CL, and Fryer AD. Ozone-induced loss of neuronal M2 muscarinic receptor function is prevented by cyclophosphamide. *J Appl Physiol* 77: 1492-1499, 1994.
- Gustín P, Dhem AR, Lomba F, Lekeux P, Van de Woestijne KP, and Landser FJ. Measurement of total respiratory impedance in calves by the forced oscillation technique. *J Appl Physiol* 64: 1786-1791, 1988.
- Hartert TV and Peebles RS Jr. Epidemiology of asthma: the year in review. *Curr Opin Pulm Med* 6: 4-9, 2000.
- Hill RH Jr, Head SL, Baker S, Gregg M, Shealy DB, Bailey SL, Williams CC, Sampson EJ, and Needham LL. Pesticide residues in urine of adults living in the United States: reference range concentrations. *Environ Res* 71: 99-108, 1995.
- Hoppin JA, Umbach DM, London SJ, Alavanja MC, and Sandler DP. Chemical predictors of wheeze among farmer pesticide applicators in the Agricultural Health Study. *Am J Respir Crit Care Med* 165: 683-689, 2002.
- Huff RA, Corcoran JJ, Anderson JK, and Abou-Donia MB. Chlorpyrifos oxon binds directly to muscarinic receptors and inhibits cAMP accumulation in rat striatum. *J Pharmacol Exp Ther* 269: 329-335, 1994.
- Jacoby DB and Fryer AD. Virus-induced airway hyperresponsiveness—possible involvement of neural mechanisms. *Am Rev Respir Dis* 144: 1422-1423, 1991.
- Jett DA, Abdallah EA, El-Fakahany EE, Eldefrawi ME, and Eldefrawi AT. The high affinity activation by paraoxon of a muscarinic receptor subtype in rat brain striatum. *Pest Biochem Physiol* 39: 149-157, 1991.
- Jett DA, Fernando JC, Eldefrawi ME, and Eldefrawi AT. Differential regulation of muscarinic receptor subtypes in rat brain regions by repeated injections of parathion. *Toxicol Lett* 73: 33-41, 1994.



40. Jett DA, Hill EF, Fernando JC, Eldefrawi ME, and Eldefrawi AT. Down-regulation of muscarinic receptors and the m3 subtype in white-footed mice by dietary exposure to parathion. *J Toxicol Environ Health* 39: 395–415, 1993.
41. Jones SM, Burks AW, Spencer HJ, Lensing S, Roberson PK, Gandy J, and Helm RM. Occupational asthma symptoms and respiratory function among aerial pesticide applicators. *Am J Ind Med* 43: 407–417, 2003.
42. Katz EJ, Cortes VI, Eldefrawi ME, and Eldefrawi AT. Chlorpyrifos, parathion, and their oxons bind to and desensitize a nicotinic acetylcholine receptor: relevance to their toxicities. *Toxicol Appl Pharmacol* 146: 227–236, 1997.
43. Katz LS and Marquis JK. Modulation of central muscarinic receptor binding in vitro by ultralow levels of the organophosphate paraoxon. *Toxicol Appl Pharmacol* 101: 114–123, 1989.
44. Katz LS and Marquis JK. Organophosphate-induced alterations in muscarinic receptor binding and phosphoinositide hydrolysis in the human SK-N-SH cell line. *Neurotoxicology* 13: 365–378, 1992.
45. Koch D, Lu C, Fisker-Andersen J, Jolley L, and Fenske RA. Temporal association of children's pesticide exposure and agricultural spraying: report of a longitudinal biological monitoring study. *Environ Health Perspect* 110: 829–833, 2002.
46. Kogevinas M, Anto JM, Sunyer J, Tobias A, Kromhout H, and Burney P. Occupational asthma in Europe and other industrialised areas: a population-based study. European Community Respiratory Health Survey Study Group. *Lancet* 353: 1750–1754, 1999.
47. Konieczny B, Kossmann S, and Makuch M. Impaired respiratory muscle function in chemical plant workers producing chlorfenvinphos. *Ann Agric Environ Med* 6: 21–25, 1999.
48. Landrigan PJ, Claudio L, Markowitz SB, Berkowitz GS, Brenner BL, Romero H, Wetmur JG, Matte TD, Gore AC, Godbold JH, and Wolff MS. Pesticides and inner-city children: exposures, risks, and prevention. *Environ Health Perspect* 107, Suppl 3: 431–437, 1999.
49. Landrigan PJ, Schechter CB, Lipton JM, Fahs MC, and Schwartz J. Environmental pollutants and disease in American children: estimates of morbidity, mortality, and costs for lead poisoning, asthma, cancer, and developmental disabilities. *Environ Health Perspect* 110: 721–728, 2002.
50. Lu C, Knutson DE, Fisker-Andersen J, and Fenske RA. Biological monitoring survey of organophosphorus pesticide exposure among preschool children in the Seattle metropolitan area. *Environ Health Perspect* 109: 299–303, 2001.
51. Maeda A, Kubo T, Mishina M, and Numa S. Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes. *FEBS Lett* 239: 339–342, 1988.
52. Manabe N, Foldes FF, Torocsik A, Nagashima H, Goldiner PL, and Vizi ES. Presynaptic interaction between vagal and sympathetic innervation in the heart: modulation of acetylcholine and noradrenaline release. *J Auton Nerv Syst* 32: 233–242, 1991.
53. Mileson BE, Chambers JE, Chen WL, Dettbarn W, Ehrlich M, Eldefrawi AT, Gaylor DW, Hamernik K, Hodgson E, Karczmar AG, Padilla S, Pope CN, Richardson RJ, Saunders DR, Sheets LP, Sultatos LG, and Wallace KB. Common mechanism of toxicity: a case study of organophosphorus pesticides. *Toxicol Sci* 41: 8–20, 1998.
54. Minette PA and Barnes PJ. Prejunctional inhibitory muscarinic receptors on cholinergic nerves in human and guinea pig airways. *J Appl Physiol* 64: 2532–2537, 1988.
55. Minette PA, Lammers JW, Dixon CM, McCusker MT, and Barnes PJ. A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. *J Appl Physiol* 67: 2461–2465, 1989.
56. Norel X, Angrisani M, Labat C, Gorenne I, Dulmet E, Rossi F, and Brink C. Degradation of acetylcholine in human airways: role of butyrylcholinesterase. *Br J Pharmacol* 108: 914–919, 1993.
57. Nostrandt AC, Padilla S, and Moser VC. The relationship of oral chlorpyrifos effects on behavior, cholinesterase inhibition, and muscarinic receptor density in rat. *Pharmacol Biochem Behav* 58: 15–23, 1997.
58. Oberhauser V, Schwertfeger E, Rutz T, Beyersdorf F, and Rump LC. Acetylcholine release in human heart atrium: influence of muscarinic autoreceptors, diabetes, and age. *Circulation* 103: 1638–1643, 2001.
59. O'Malley M. Clinical evaluation of pesticide exposure and poisonings. *Lancet* 349: 1161–1166, 1997.
60. Overstreet DH. Organophosphate pesticides, cholinergic function and cognitive performance in advanced age. *Neurotoxicology* 21: 75–81, 2000.
61. Perera FP, Illman SM, Kinney PL, Whyatt RM, Kelvin EA, Shepard P, Evans D, Fullilove M, Ford J, Miller RL, Meyer IH, and Rauh VA. The challenge of preventing environmentally related disease in young children: community-based research in New York City. *Environ Health Perspect* 110: 197–204, 2002.
62. Pope CN, Chakraborti TK, Chapman ML, and Farrar JD. Long-term neurochemical and behavioral effects induced by acute chlorpyrifos treatment. *Pharmacol Biochem Behav* 42: 251–256, 1992.
63. Pope CN, Chakraborti TK, Chapman ML, Farrar JD, and Arthun D. Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. *Toxicology* 68: 51–61, 1991.
64. Schuh RA, Lein PJ, Beckles RA, and Jett DA. Noncholinesterase mechanisms of chlorpyrifos neurotoxicity: altered phosphorylation of Ca<sup>2+</sup>/cAMP response element binding protein in cultured neurons. *Toxicol Appl Pharmacol* 182: 176–185, 2002.
65. Schwartz DA. Etiology and pathogenesis of airway disease in children and adults from rural communities. *Environ Health Perspect* 107, Suppl 3: 393–401, 1999.
66. Segura P, Chavez J, Montano LM, Vargas MH, Delaunois A, Carbajal V, and Gustin P. Identification of mechanisms involved in the acute airway toxicity induced by parathion. *Naunyn-Schmiedeberg's Arch Pharmacol* 360: 699–710, 1999.
67. Senthilselvan A, McDuffie HH, and Dosman JA. Association of asthma with use of pesticides. Results of a cross-sectional survey of farmers. *Am Rev Respir Dis* 146: 884–887, 1992.
68. Silveira CL, Eldefrawi AT, and Eldefrawi ME. Putative M2 muscarinic receptors of rat heart have high affinity for organophosphorus anticholinesterases. *Toxicol Appl Pharmacol* 103: 474–481, 1990.
69. Sirajuddin H, Roslinah A, Rampal KG, Kuppusamy I, Rohna R, Aziz M, Aw TC, and Beach JR. Notification of occupational and work-related diseases and poisonings in Malaysia, 1997–1998. *Med J Malaysia* 56: 25–31, 2001.
70. Stanton ME, Mundy WR, Ward T, Dulchinos V, and Barry CC. Time-dependent effects of acute chlorpyrifos administration on spatial delayed alternation and cholinergic neurochemistry in weanling rats. *Neurotoxicology* 15: 201–208, 1994.
71. USDA. Agricultural chemicals and production technology. Washington, DC: Economic Research Service, 2003.
72. Ward TR and Mundy WR. Organophosphorus compounds preferentially affect second messenger systems coupled to M2/M4 receptors in rat frontal cortex. *Brain Res Bull* 39: 49–55, 1996.
73. Weisenburger DD. Human health effects of agrichemical use. *Hum Pathol* 24: 571–576, 1993.
74. Whyatt RM, Camann DE, Kinney PL, Reyes A, Ramirez J, Dietrich J, Diaz D, Holmes D, and Perera FP. Residential pesticide use during pregnancy among a cohort of urban minority women. *Environ Health Perspect* 110: 507–514, 2002.
75. Wilhoit L, Davidson N, Supkoff D, Stegall J, Braun A, Simmons S, Hobza B, Hunter C, Gorder N, Goodman C, and Todd B. Pesticide Use Analysis and Trends from 1991 to 1996. Sacramento, CA: State of California Environmental Protection Agency, 1999.
76. Zhang LX, Enarson DA, He GX, Li B, and Chan-Yeung M. Occupational and environmental risk factors for respiratory symptoms in rural Beijing, China. *Eur Respir J* 20: 1525–1531, 2002.